CHANGE IN DEHYDRODOLICHYL DIPHOSPHATE SYNTHASE DURING SPERMATOGENESIS IN THE RAT

BY

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This dissertation is dedicated to my parents, Quo-Wei Chen and Zhi-Huei Chang, my loving wife, He-Ping Han, and my son, Henry Hai Pei.

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KEY TO ABBREVIATIONS

BSA	bovine serum albumin
Ci	curie
cm	centimeter
cpm	counts per minute
°C	degree centigrade
DIBK	diisobutyl ketone
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DOL	dolichol
DOL P	dolichyl phosphate
DOL PP	dolichyl diphosphate
dpm	disintegrations per minute
EKRB	enriched Krebs-Ringer bicarbonate medium
IPP	isopentenyl diphosphate
FPP	t,t-farnesyl diphosphate
Glc	glucose
GlcNAc	acetylglycosamine
GPP	geranyl diphosphate
g	gram
HAc	acetic acid
hr	hour
M	molar

Man	mannose
MEM	Eagle's minimal essential medium
μ	micron
μCi	microcurie
μg	microgram
μl	microliter
μM	micromolar
μmole	micromole
mM	millimolar
mm	millimeter
mmole	millimole
PBS	phosphate buffered saline
pmole	picomole
PP	diphosphate
RNA	ribonucleic acid
rpm	revolutions per minute
TLC	thin layer chromatography
Tris	Tris-(hydroxymethyl)aminomethane
v/v	on a volume-to-volume basis
v/w	on a volume-to-weight basis
w/v	on a weight-to-volume basis
w/w	on a weight-to-weight basis

Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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Dolichyl phosphate (DOL P), a carbohydrate carrier in glycoprotein biosynthesis, may be formed from the metabolism of dehydro dolichyl diphosphate (dehydro DOL PP). A method has been developed to measure 2,3-dehydro DOL PP synthase with [^{14}C]-isopentenyl diphosphate and t,t-farnesyl diphosphate. Enzymatic activity was measured in homogenates prepared by sonication of seminiferous tubules or isolated cell fractions. There was a 2 fold increase in tubular activity between day 7 and day 23 and a similar decrease in activity between day 23 and day 60. The increase in activity paralleled an increase in DOL P concentration, suggesting a regulatory role for the synthase in DOL P synthesis.

The activity in homogenates of protease treated seminiferous tubules, and cell fractions enriched in spermatogenic cells or Sertoli cells also changed as a function of age in the rats. The highest enzymatic activity occurred in each case at age 23 days. Cell fractions enriched in pachytene spermatocytes, spermatids or Sertoli cells were shown to have higher synthase activity than a whole testicular homogenate or a mixture of cells prepared by collagenase-trypsin treatment of tubules. Enzymatic activity in pachytene spermatocytes expressed as pmoles/mg protein was about 5.3 fold higher than spermatogonia, 1.7 fold higher than spermatids and about 8.3 fold higher than spermatozoa. The enzymatic activity of pachytene spermatocytes expressed as pmoles/10^6 cells was 4.5 fold higher than spermatids and about 126 fold higher than spermatozoa. These studies are the first to show that Sertoli cells have the potential to synthesize DOL.

The increase in synthase activity in spermatogenic cells and Sertoli cells during early stages of spermatogenesis indicates that both cell populations are contributing to the increase in activity in seminiferous tubules. Furthermore, the increase can be explained on the basis of changes in the specific activities of the Sertoli cells and the different populations of spermatogenic cells. This increase may be important in regulating the availability of dolichyl phosphate for glycoprotein biosynthesis during early stages of spermatogenesis.

CHAPTER I
INTRODUCTION

Spermatogenesis

Spermatogenic Cycles and Waves

Spermatogenesis is a developmental process in which the spermatogenic cell undergoes a series of biochemical and morphological changes through three well described phases: spermatogonial renewal and proliferation, meiosis, and spermiogenesis. Spermatogenesis in the rat starts during fetal development with appearance of gonocytes by postnatal day 4 and continues throughout adult life. The first spermatozoa appear within the lumens of the seminiferous tubules at about 45 days of ages, and all stages of spermatogenic cycle are represented (Clermont & Perey, 1957; Knorr et al., 1970). The initial phase of spermatogenesis, the spermatogonial phase, occurs in the basal compartment of the seminiferous epithelium and consists of a mitotic proliferation of spermatogonia from stem cells. The spermatogonia divide and differentiate sequentially into type A spermatogonia, intermediate spermatogonia, and type B spermatogonia. The type B spermatogonia divide to form

preleptotene primary spermatocytes, which undergo a final replication of nuclear DNA before entering meiotic prophase. The preleptotene spermatocytes migrate from the basal compartment to the adluminal compartment where spermatogenesis is completed. The second phase of spermatogenesis, meiosis, occurs while the spermatocytes remain on the adluminal side of the intercellular Sertoli junctions. Meiotic prophase, which is subdivided into the stages of the leptotene, zygotene, pachytene, diplotene, and diakinesis, terminates in the first meiotic, or reductional, division with the formation of secondary spermatocytes. The latter cells quickly enter the second meiotic, or equational division to form the haploid spermatids. Spermiogenesis, the final phase of spermatogenesis, consists of a complex morphological transformation of the haploid spermatogenic cell, that culminates with the release of late spermatids into the lumen of the seminiferous tubule.

Spermatogenesis has many unique features. The most remarkable ones include the spermatogenic cycles and waves in the seminiferous epithelium. The various generations of spermatogenic cells are not randomly distributed in the seminiferous epithelium, but are organized into well-defined cellular associations. Certain cells are always found in association with certain other cells. Each of these cells develops in synchrony with the others, so that if we could watch one section of the tubule wall with a time-lapse

camera, a series of different cell associations would be seen, until the cycle was completed. The time interval between the appearance of the same cell association at a given point of the tubule is called the cycle of the seminiferous epithelium. The number of stages in the cycle is constant for a given species; man has 6, mouse and monkey have 12, the rat, 14 (Figure 1-1).

The cycle involves changes with time in the appearance of one segment of a tubule, whereas the wave refers to the distribution of different cellular associations along the length of the tubule. The segments of the tubules which specific cellular associations occur in a sequential series along the length of the tubule. For example a segment containing cells at stage V of the cycle is bordered on one side by cells at stage IV and on the opposite side is at stage VI. The average length of each tubular segment correlates roughly with the relative duration of the corresponding cellular association or stage of the cycle. The sequence of segments or waves, representing the stages in the cycle of the seminiferous epithelium, repeats itself along the length of an individual tubule. In the rat, for example, there is an average of 12 waves per tubule.

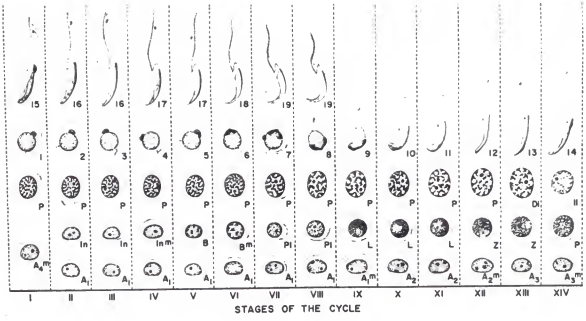


Figure 1-1. The Cellular Composition of the 14 Stages of the Cycle of the Seminiferous Epithelium in Rat.

Each numbered column (roman numeral) shows the spermatogenic cell types present in cellular associations found in cross sections of seminiferous tubules. The cellular associations or stages of the cycle succeed one another in time in any given area of the seminiferous epithelium in the rat. Following cellular association XIV, cellular association I reappears, so that the sequence start over again. Steps in the development of the spermatids, numbered 1 to 19 and defined by changes in the structure of the nucleus and acrosome, are indicated with arabic numerals. Letters are used to identify spermatogonia and spermatocytes. A_1, A_2, A_3, and A_4, represent four generations of type A spermatogonia; In, intermediate spermatogonia; B, type spermatogonia; Pl, preleptotene spermatocytes; L, leptotene spermatocytes; Z, zygotene spermatocytes; P, pachytene spermatocytes; Di, Diplotene and Diakinesis; II, secondary spermatocytes. The subscript m indicates the occurrence of mitotic division of the spermatogonia (From Dym, M. & Clermont, Y., 1970, Am. J. Anat., 128, 265-282,).

Exocrine and Endocrine Functions of Testis

The testis has both exocrine and endocrine functions. The exocrine function of the testis resides in the cells of the seminiferous epithelium which produce testicular fluids and spermatozoa. The endocrine function of the testis resides primarily in the Leydig cell population which synthesizes and secretes the principal circulating androgen, testosterone (Nearly 95% of the testosterone is produced by the testis; the rest is produced by the adrenal glands). The steroidogenic and spermatogenic activities of the testis are regulated by hormonal interactions among the hypothalamus, adenohypophysis, and the cells of the gonad — the Sertoli cells, spermatogenic, and Leydig cells.

The Leydig cells, which are located in the space surrounding the seminiferous tubules, start to secrete testosterone during the fetal period, promoting the development of the male reproductive tract.

Biosynthesis of Glycoproteins During Spermatogenesis in Testis

Mammalian spermatogenesis involves extensive morphological and biochemical transformations to produce mature gametes that are structurally and functionally unique. During this process, gene expression is temporally regulated both at transcriptional and translational levels (Bellve & O'Brien, 1983; Hecht, 1986). A variety of proteins, many of which are unique to spermatogenic cells, are differentially

expressed during meiosis and spermiogenesis. These include basic chromosomal proteins that undergo successive transitions during spermatogenic cell differentiation (Meistrich et al., 1981), several spermatogenic cell-specific isozymes (Goldberg, 1977) and stage-specific surface glycoproteins identified with biochemical (Millette & Moulding, 1981a; Millette & Moulding, 1981b) and immunological probes (O'Rand & Romrell, 1980; Gaunt, 1982; Fenderson et al., 1984; O'Brien & Millette, 1984; O'Brien & Millette, 1986). It has been suggested that many proteins play roles in various processes including sperm-egg interaction, Sertoli-spermatogenic cell association, capacitation and the acrosomal reaction. For example, rabbit sperm autoantigen (RSA-1), a sialoglycoprotein located in the postacrosomal region, plays a role in the spermatozoon's binding to and penetration of the zona. This protein first appears on the surface of pachytene spermatocytes and increases in amount throughout spermatogenesis (O'Rand & Romrell, 1981; O'Rand et al., 1984). Such sperm specific surface components are probably involved in antibody mediated agglutination and immobilization of spermatozoa.

Early in spermatogenesis, glycoproteins are synthesized and deposited in the acrosome. One of the acrosomal glycoproteins is proacrosin, which yields acrosin after being activated. Proacrosin is first produced during the spermatid stage of differentiation, and is retained throughout the

remainder of spermatogenesis (Florke et al., 1983). Acrosin is thought to be an essential protease required for the proteolysis of the zona pellucida of the ovum during fertilization (Hartree, 1977). In purified rabbit proacrosin, glucosamine, mannose, galactose and sialic acid were found in the ratio of 3,3,1,1 per mole of proacrosin which is consistent with the ratio expected of these sugars in N-linked glycoproteins (Mukerji & Meizel, 1979). Therefore, proacrosin would be expected to be synthesized via a pathway which involved dolichyl phosphate (DOL P) metabolism.

A general study of the glycosylation of protein would be of value in understanding factors which might regulate the biosynthesis of these cell and stage specific proteins. An understanding of the origin, metabolic pathway and mechanism of biosynthesis of these glycoproteins during spermatogenesis may provide insight into the biochemical control of the cell function and differentiation. These findings may be of significance in the design of male contraceptive agents and in understanding molecular basis of male sterility.

Sertoli Cell

Histological Structures and Functions

The Sertoli cells are the nongerminal elements in the seminiferous tubules of the testes. They were first

described in 1865 by Sertoli to have a nursing function, to provide mechanical support for the developing spermatogenic cells as well to be phagocytic. It is believed that circulating hormones, which act on spermatogenesis, have their effects mediated via the Sertoli cell (Hansson et al., 1976).

Sertoli cells are basically columnar cells, which surround the adjacent spermatogenic cells and fill the spaces between them (Figure 1-2). Sertoli cells form the major structural component of the seminiferous tubules and serve a number of functions, including (a) mediating movement of spermatogenic cells from the basal lamina to the lumen and the release of the late spermatids into the tubular lumen, (b) compartmentalizing the epithelium into basal and adluminal compartments and forming part of the blood-testis barrier, (c) phagocytizing degenerating spermatogenic cells and residual bodies, (d) secreting androgen binding protein and inhibin as well as other glycoproteins and (e) mediating the movement of steroids, metabolites, and nutrients utilized by spermatogenic cells across the seminiferous epithelium.

During development, the Sertoli cell undergoes fundamental changes. The most remarkable feature of these maturational changes is the cessation of cell multiplication that occurs before puberty. It occurs in the rat at about 15 days of age (Steinberger & Steinberger, 1971). After that age, the Sertoli cells may change their metabolic activities

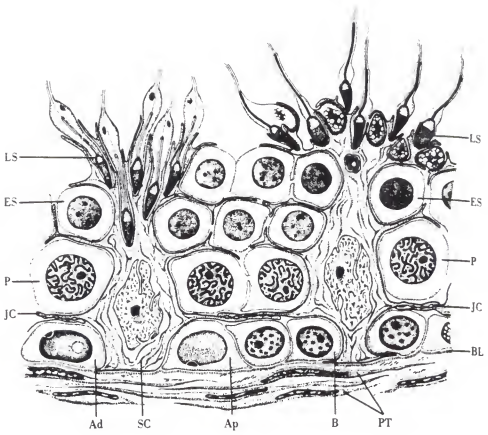


Figure 1-2. Schematic Drawing of Human Seminiferous Epithelium.

The seminiferous epithelium recline upon a basal lamina (BL), and a layer of peritubular cells (PT) surrounds the seminiferous tubule. Pale type A spermatogonium (Ap), dark type A spermatogonium (Ad), and type B spermatogonium are located in the basal compartment of the seminiferous epithelium below the junctional complex (JC) between adjacent Sertoli cells (SC); pachytene primary spermatocytes (P), early spermatids (ES), and late spermatids (LS) are seen in the adluminal compartment above the junctional complex (Ross, M. H. and Reith, E. J., 1985, Histology, 3rd printing, pp 608; Clermont, Y. 1963, Am. J. Anat., 112, 35).

under the influence of various factors, but they do not divide any more.

Adjacent Sertoli cells are joined by the Sertoli cell junctional complex, which is a unique structure not found in other epithelium tissues. This functional barrier develops in the rat at about 16 to 19 days of age (Vitale et al., 1973). The Sertoli-Sertoli junctional complex divides the seminiferous epithelium into two compartments: the basal compartment and the adluminal compartment. Actually, the Sertoli-Sertoli junctional complexes are the site of the blood-testis barrier, which serves an essential role in isolating the spermatogenic cells from the immune system; i.e. the production of unique molecules on spermatogenic cells is recognized as foreign if these molecules come in contact with the immune system.

Follicle stimulating hormone (FSH) and testosterone regulate the process of sperm production within the seminiferous epithelium. The Sertoli cells have been shown to be the primary target for FSH and androgens. Therefore, the Sertoli cells are considered to be the regulators of spermatogenesis. The probable importance of Sertoli cells in spermatogenesis has been emphasized by a number of investigators (Bridges et al., 1986; Fritz et al., 1976; Griswold et al., 1986); however, their precise role in this process is still not fully understood.

Physiological and morphological studies have indicated that the Sertoli cells undergo cyclic changes in their metabolic activity which are related to specific stages in the cycle of the seminiferous epithelium. Cytochemical studies showed that several enzyme activities in Sertoli cells vary depending on the stage of the cycle of the seminiferous epithelium. For instance, the peak activity of acid phosphatase appeared at stages VII and VIII, but little activity was found in stages IX through II (Niemi & Kormano, 1965). A similar stage specific distribution has been observed with the thiamine pyrophosphatase in Sertoli cells (Hilscher et al., 1979).

Sertoli Cells Secrete Many Glycoproteins

One of the mechanisms by which Sertoli cells biochemically influence the spermatogenic cells is through the synthesis and secretion of glycoproteins. At least 15% of all the proteins synthesized by Sertoli cell are glycoproteins (Bridges et al., 1986). Some of the glycoproteins secreted by the Sertoli cells are specific to the testis, and others are similar, if not identical, to serum proteins. Sertoli cells secrete glycoproteins into the lumen of the tubules, perhaps into the blood stream or lymph, and maybe into the space between Sertoli cells and spermatogenic cells for subsequent uptake by spermatogenic cells. Several glycoproteins secreted by the Sertoli cells

have been studied, such as androgen binding protein (ABP) (Fritz et al., 1976), plasminogen activator (Lacroix et al., 1977), testicular transferrin (Skinner & Griswold, 1980), and sulfated glycoprotein 1 (SPG-1) and 2 (SPG-2) (Griswold et al., 1986).

The stage specific nature of the elaboration of one glycoprotein, androgen binding protein, has been particularly well described. Sertoli cells have maximum FSH binding during stages XIII through I followed by maximum cAMP production in stages II through VI which in turn is followed by maximum production of androgen binding protein in stages VII through VIII (Parvinen et al., 1980; Ritzen et al., 1982). Since Sertoli cells are actively involved in glycoprotein biosynthesis, we speculate that the regulation of the N-linked glycoprotein biosynthesis may be significant in the function of these cells.

Role of DOL P in Glycoprotein Biosynthesis

The Structure and Distribution of Dolichol and Its Derivatives

Dolichol (DOL) is a general term for a group of polyisoprenoid alcohols. They contain the dimethylallyl terminal unit (ω-terminal), two *trans*-isoprene residues, a number of *cis*-isoprene residues and a terminal hydroxylated α-saturated isoprene unit linked in a head-to-tail orientation. Pennock, Hemming and Morton first isolated and

characterized this compound from pig liver (Pennock et al., 1960). Mammalian polyprenols contain a larger number of isoprene residues C_{85}-C_{115} (17-23 x C_5) than those present in plants and bacteria C_{50}-C_{60} (10-12 x C_5). Polyprenols found in bacteria have an α-unsaturated isoprene unit. However, it is known that liver contains small amounts of α-saturated shorter polyprenols (Mankowski et al., 1976), and in some tissues, such as bovine pituitary gland (Rodominska-Pyrek et al., 1979) and hen oviduct (Hayes & Lucas, 1980), α-unsaturated compounds have been reported. DOL is present in most eukaryotic cells and is found in particularly high concentration in some human tissues, such as the adrenal gland, pancreas, pituitary gland, testis and thyroid gland (Rupar & Carroll, 1978; Tollbom & Dallner, 1986). James and Kandutsch showed that in mouse the synthesis of DOL is much more active in testes than in liver (James & Kandutsch, 1980c). It seems that DOL content is relatively high in the rapidly growing and differentiating tissues. For example, the DOL content of the hyperplastic liver nodules in liver is four times higher in the homogenate and six times higher in the microsomes than that found in normal rat liver. In developed hepatocarcinoma, the amount of DOL was found to be doubled. In contrast to free DOL, DOL P was found to be greatly decreased in nodules (Eggens et al., 1984).

Other studies show an increase in DOL levels with age although the increases vary widely with tissues (brain,

liver, kidney, testis, lung and heart) (Pullarkat et al., 1984).

DOL may have some effects on membrane structure and fluidity (Valtersson et al., 1985). Otherwise, most DOL apparently has no direct relationship to glycoprotein synthesis, since the bulk of the DOL is present in membranes other than the endoplasmic reticulum (Wong et al., 1982; Adair & Keller, 1982; Ekstrom et al., 1982). Some DOL may be phosphorylated by a CTP-dependent kinase (Allen et al., 1978; Burton et al., 1979). Dolichol is found in several combined forms in the cell, free dolichol and esterified form with fatty acids or mono or diphosphate. The phosphorylated forms are important as we will see soon because they are also found with different degrees of glycosylation which is important for glycoprotein biosynthesis.

Dolichyl fatty acyl esters may have several metabolic roles. First, the fatty acyl esters may be a suitable and stable form for the storage of dolichol in lipid droplets. Alternatively, the fatty acyl moiety might be necessary for the transport of dolichol from its site of synthesis to different subcellular locations.

The percentage of dolichol found in the esterified form varies widely in different tissue. For example, it is reported to be 0% and 25% esterified in pig kidney and spleen, respectively. In another report, it is shown that the ester form represents about 63% of total DOL in pig

liver, and 65-90% in mouse testes and preputial glands (Malvar et al., 1985). The mechanism which determines these distributions is not known.

DOL P in Glycoprotein Synthesis

In the early 1970s, Behrens and Leloir demonstrated the involvement of DOL P in the N-linked glycoprotein biosynthesis (Behrens & Leloir, 1970; Behrens et al., 1971). Although, only a small amount of the total DOL in cells is phosphorylated (Eggens et al., 1983), the phosphorylated form (DOL P) is an essential carbohydrate carrier in the biosynthesis of asparagine-linked glycoproteins (Struck & Lennarz, 1980; Hubbard & Ivatt, 1981).

Most secretory proteins and membrane proteins, numerous receptors and proteins related to cell recognition are glycoproteins. A typical N-linked glycoprotein contains one or a few oligosaccharide units linked to asparagine side chains by N-glycosidic bonds.

The biosynthesis of N-linked glycoproteins proceeds through a cyclic process, which requires the involvement of DOL P, in the synthesis of the core oligosaccharide unit (Parodi & Leloir, 1979; Hubbard & Ivatt, 1981).

Figure 1-3 represents a highly abbreviated representation of this cyclic process and associated reactions (Rip et al., 1985; Hemming, 1985, for review).

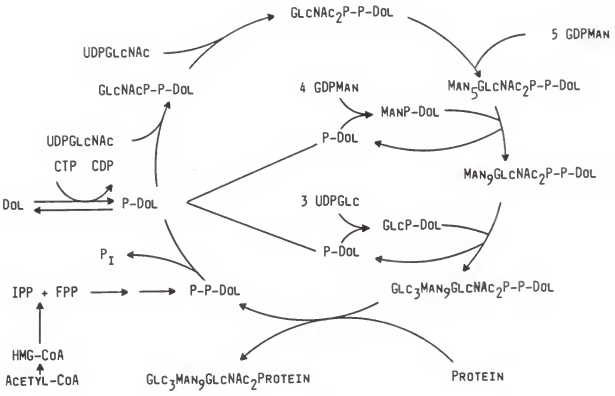


Figure 1-3. Dolichol cycle for glycoprotein formation in eukaryotic cells.

The first step in the assembly of lipid-linked oligosaccharide involves the addition of GlcNAc-P from UDP-GlcNAc to DOL P to generate DOL PP-GlcNAc. Then, this molecule reacts with an additional UDP-GlcNAc to form DOL PP-$(GlcNAc)_2$. Five mannose residues are added next from GDP-mannose to form DOL PP-$(GlcNAc)_2$-Man_5. It had been shown that DOL P-Man also is a mannose donor to the core oligosaccharide chain. In the oligosaccharide lipid carrying nine mannose residues, the last four of these are transferred through DOL P (Rearick et al., 1981). The next steps are transfers of three glucose units from DOL P-Glc to the core oligosaccharide chain with the formation of the lipid-linked oligosaccharide DOL PP-$(GlcNAc)_2$-Man_9-Glc_3. In the final step of this pathway, the core oligosaccharide is transferred en bloc to newly synthesized polypeptide with the concomitant release of DOL PP. This core protein linked oligosaccharide is then processed by a now well described pathway involving specific glycosidases (Kornfeld & Kornfeld, 1980) and additional sugar residues added by DOL P independent glycosyl transferases. Schachter and his coworkers from studies of glycoprotein and glycolipid metabolism during spermatogenesis in rat and mouse testis indicated that spermatocytes and early spermatids had highly active glycosylating systems (Kornblatt et al., 1974; Letts et al., 1974a; Letts et al., 1974b).

DOL P plays a major role in the biosynthesis of N-linked glycoprotein, since DOL P not only is an oligosaccharide unit carrier but also is an activator which reacts with certain nucleotide sugars and facilitates the sugar transfer to the core oligosaccharide chains. The involvement of DOL P in the production of the DOL PP-oligosaccharide and the subsequent transfer of the oligosaccharide to asparagine residues within the newly formed peptide is thought to occur on the luminal surface of the endoplasmic reticulum membrane (Pfeffer & Rothman, 1987, for review).

Studies with chick embryo fibroblast (Hubbard & Robbins, 1980) and canine kidney cells (Schmitt & Elbein, 1979) suggest that oligosaccharide transfer is the rate limiting step in glycoprotein synthesis. Subsequent regeneration of DOL P permits the reinitiation of lipid oligosaccharide biosynthesis. Therefore, enzymes of the DOL P pathway associated with formation or utilization of DOL P could be very important in the control of glycoprotein biosynthesis in spermatogenesis.

The availability of DOL P was found to be a rate-limiting factor in some glycosylation processes (Potter et al., 1981a; Eggens et al., 1984). Furthermore, the shortage of the lipid intermediates influences some vital biological processes such as embryonic development (Carson et al., 1981). Carson and Lennarz showed that when DOL P synthesis

was inhibited by compactin, a potent inhibitor of hydroxymethyl glutaryl CoA reductase and consequently polyisoprenoid biosynthesis, protein glycosylation was impaired and the oligosaccharide chains synthesized were more negatively charged (Carson & Lennarz, 1981). In another report, inhibition of DOL P biosynthesis induced abnormal gastrulation in sea urchin embryos (Carson & Lennarz, 1979). All of the findings suggested that DOL P plays an important role in the N-linked glycoprotein biosynthesis. Needless to say, a good understanding in the regulation of DOL metabolism is a necessary step to explore the regulation of the N-linked glycoprotein biosynthesis.

Metabolism and Functions of DOL P

There are at least three pathways to generate DOL P. First, the dephosphorylation of DOL PP could provide the main supply of DOL P for the biosynthesis of intermediates in the protein glycosylation reactions (Dallner & Hemming, 1981). On the other hand, investigations in recent years have established that increased protein glycosylation is often accompanied by increased phosphorylation of DOL by a CTP-dependent kinase; therefore, a second possibility is that DOL P is supplied by direct phosphorylation of DOL (Burton et al., 1981; Coolbear & Mookerjea, 1981). Third, when the sugar residues are transferred to the growing oligosaccharide chain, DOL P is released from DOL P-Man or DOL P-Glc.

Since DOL P is an important precursor of both DOL P-monosaccharide and DOL P-oligosaccharide, it is understandable that a shortage of DOL P could have multiple effects on the biosynthesis of lipid-oligosaccharide and may cause the production of defective glycoproteins. Chapman has shown that a mouse lymphoma cell mutant, lacking DOL P, can not synthesize DOL P-Man (Chapman et al., 1980). Kean (1985), using microsomes from a variety of tissues, reported that DOL P-Man, which requires DOL P for its biosynthesis, exerts a positive allosteric effect on the enzymes that catalyze the formation of DOL PP-GlcNac. More recently, Carson et al. (1987) found that during hormonal induction of glycoprotein assembly in mouse uteri, the changes in the rate of DOL P-Man synthesis may be an important factor in regulating DOL P-linked oligosaccharide assembly, since uteri contain very high levels of DOL P and DOL P linked saccharides.

DOL PP, in turn, can arise via two metabolic pathways. First, by a recycling mechanism, where DOL PP is released from the lipid oligosaccharide when the oligosaccharide portion is transferred to the newly synthesized polypeptide. Alternatively, DOL PP may be derived from de novo biosynthesis by a poorly defined pathway which undoubtedly requires the condensation of low molecular weight precursors, such as farnesyl diphosphate and isopentenyl diphosphate. Regulation of this pathway could be very important for

controlling DOL P level in the cells, since this pathway is the only de novo biosynthesis pathway known and serve as a "bridge" connecting the small metabolites, such as acetyl CoA, with the large DOL molecules. A key enzyme of this pathway is dehydro DOL PP synthase, which catalyzes the synthesis of dehydro DOL PP from farnesyl diphosphate and isopentenyl diphosphate. Dehydro DOL PP synthase could be an important cellular regulator of glycoprotein biosynthesis as a consequence of its regulation in the DOL P de novo biosynthesis. A postulated pathway showing the important role of dehydro DOL PP synthase in DOL P and glycoprotein biosynthesis is demonstrated in Figure 1-4. However, this enzyme has not been well studied.

One approach to clarifying the fate of DOL in vivo has been to inject this compound into an experimental animal and thereafter monitor its appearance in various organs. After injection of ^{3}H-DOL into the bloodstream of a rat, the radioactivity rapidly appeared in the high density lipoprotein (HDL) fraction of blood, with subsequent uptake into all tissues (Keenan et al., 1977). Since Elmberger suggested that rat liver might be the main or exclusive site of DOL synthesis, the presence of DOL in the high density lipoprotein fraction of blood may point to high density lipoprotein as a DOL transporter (Elmberger et al., 1987).

The rate of clearance of ^{14}C-DOL from tissues of the rat is very slow, since about half the radioactivity is

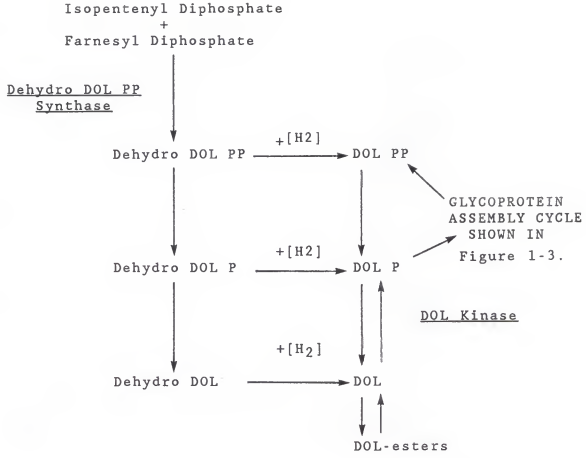


Figure 1-4. A putative pathway showing the relationship between dehydro DOL PP and glycoprotein biosynthesis.

present in the whole rat and in the liver 24 hr after injection. Furthermore, it was still present 20 days later, almost entirely as ^{14}C-DOL. The half-life of DOL P in rat liver has been estimated to be 7-12 days on the basis of the size of the DOL P pool. The catabolic products derived from labeled DOL have not yet been found (Rip et al., 1985).

The Importance of Dehydro DOL PP Synthase

Studies on developing brain (James & Kandutsch, 1980a), and liver (Keller et al., 1979; Tavares et al., 1981; Keller, 1986) have clearly shown that the rates of cholesterol and dolichol biosynthesis are regulated independently, although they share in part a common biosynthetic pathway.

Although 3-hydroxy-3-methylglutaryl CoA reductase (HMG CoA reductase), the rate limiting enzyme in cholesterol synthesis and whose product, mevalonate, is a metabolic precursor of DOL, was shown to be high in these cells, the fact that DOL and cholesterol biosynthesis can be independently regulated would lead to uncertainty about the potential of this enzyme as the regulatory enzyme in DOL biosynthetic pathway.

Dolichol, cholesterol and ubiquinone are all formed naturally from mevalonate. During the synthesis, mevalonate undergoes a series of reactions and is converted to isopentenyl diphosphate, which in turn, is isomerized to form dimethylallyl diphosphate. Then there is a sequential

condensation of isopentenyl diphosphate first to the dimethylallyl diphosphate primer and then to the consequential allylic diphosphate. Earlier biosynthetic experiments confirmed that the isoprene residues of polyisoprenoid alcohols are added in a stereochemically specific manner (Hemming, 1970). The cis - addition to trans, trans-farnesyl diphosphate gives rise to dolichols, and the trans-addition to trans, trans-farnesyl diphosphate generates the polyprenyl side chains of ubiquinones. On the other hand, the synthesis of squalene, a cholesterol precursor, results from a reductive condensation of two molecules of trans, trans-farnesyl diphosphate (Figure 1-5).

Many enzymes are common to the pathways of cholesterol and DOL biosynthesis. In particular HMG-CoA reductase, which is a major regulatory enzyme for cholesterol biosynthesis (Faust et al., 1979; Rodwell et al., 1976), could also control the biosynthesis of isoprene units utilized for dolichol synthesis. But, effectors that inhibit cholesterol biosynthesis in a major way have minimal effect on DOL biosynthesis (James & Kandutsch, 1980b). A current debatable hypothesis to explain these findings suggests that dehydro DOL PP synthase is saturated at a much lower concentration of the prenyl diphosphate substrates than the enzymes of cholesterol pathway, and therefore, the synthase is less subject to large changes in the availability of these substrates (Keller et al., 1979; James & Kandutsch, 1979).

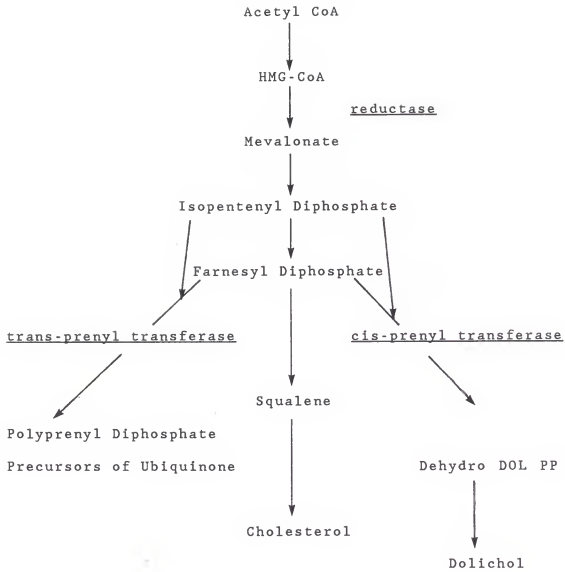


Figure 1-5. Pathway of Isoprenoid Biosynthesis. cis-prenyl transferase is the same as dehydro DOL PP synthase.

Therefore, it is likely that the pathway of DOL synthesis may have its own regulatory point which is independent of cholesterol biosynthesis. This could make dehydro DOL PP synthase a rate-limiting step in DOL biosynthesis. This, coupled with the finding that DOL synthesis in developing systems is greatly enhanced relative to cholesterol biosynthesis, makes it likely that large increases in dehydro DOL PP synthase activity might accompany or precede an increase in glycoprotein synthesis.

Studies Related to DOL P Biosynthesis and Spermatogenesis

The Role of Dehydro DOL PP in DOL Metabolism

Testicular tissues contain large quantities of DOL and are actively engaged in glycoprotein synthesis. Early studies on human tissues by Rupar and Carroll (1978) using gravimetric methods for determining DOL concentrations suggested that testis contained more DOL than any other organ.

James and Kandutsch (1980b) suggested that one or more of the spermatogenic cell types are responsible for the high rate of DOL synthesis observed in normal testicular tissue. Further studies of Potter et al. (1981b) showed that purified mouse spermatogenic cell populations are capable of DOL synthesis. The pachytene spermatocyte were the most active, whereas the round spermatids are less active. These studies

clearly demonstrated high DOL synthesis in specific spermatogenic cell populations. However, the enzyme or enzymes that might be responsible for this increased DOL synthesis were not identified.

In order to understand how glycoprotein assembly is coordinated with differentiation, it is necessary to understand how the individual steps in the sequence of glycoprotein assembly are modulated. We focus our attention here on the biosynthesis of the carbohydrate carrier, DOL PP.

It is believed that dehydro DOL PP, a derivative of DOL PP with an α-unsaturated isoprene unit, is an intermediate in the de novo biosynthesis of DOL PP. Figure 1-6 compares the structures of dehydro DOL PP and DOL PP.

The enzyme catalyzing the synthesis of this intermediate is dehydro DOL PP synthase. Dehydro DOL PP biosynthesis from isopentenyl diphosphate (IPP) and farnesyl diphosphate (FPP) has been demonstrated in hen oviduct (Grange & Adair, 1977), Ehrlich tumor cells (Adair & Trepanier, 1980), chicken liver (Wellner & Lucas, 1979), mouse L-1210 cells (Adair & Cafmeyer, 1987a) and yeast (Adair & Cafmeyer, 1987b).

$$FPP + (IPP)_{14-20} \longrightarrow \text{dehydro DOL PP } (17\text{-}23 \times C_5)$$

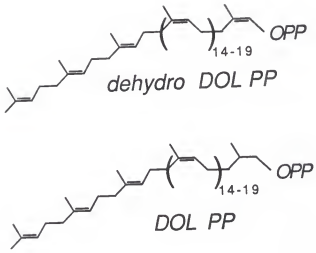


Figure 1-6. The structure of dehydro DOL PP and DOL PP. The α-unsaturated isoprene unit is present on the structure of dehydro DOL PP.

The condensation of these substrates starts a polymerization reaction which finally leads to the formation of dehydro DOL PP with farnesyl diphosphate providing the three ω-terminal isoprene units and the last isopentenyl diphosphate added providing the α-unsaturated unit bearing the diphosphate. The hen oviduct and Ehrlich tumor cell enzymes are membrane associated.

Dehydro DOL P biosynthesis from isopentenyl diphosphate and farnesyl diphosphate has also been described in microsomal fractions of rat liver (Wong & Lennarz, 1982).

Adair and Keller (1982) have showed that the enzymatic products of the liver enzyme were a group of dehydro DOL monophosphates ranging in size from C_{75} to C_{95} ($15\text{-}19 \times C_5$). Recent data from this laboratory have shown that rat testicular homogenates and their membrane fractions will catalyze the synthesis of C_{75}-C_{85} dehydro DOL P and dehydro DOL PP from t,t-farnesyl diphosphate and isopentenyl diphosphate (Baba et al., 1987). The isolation of dehydro DOL P and DOL P in some cases instead of the diphosphate analogues reflects the activity of diphosphatases.

A mechanism of reduction of dehydro dolichyl derivatives to DOL was suggested recently by Ekstrom et al. (1987). They hypothesized that during the saturation of the terminal isoprene unit, dehydro DOL PP (also referred to by some as polyprenols to distinguish them from the saturated counterparts, the dolichols) are elongated by an additional isoprene residue and saturated at the same time.

The enzymes of DOL metabolism are membrane associated and, therefore, most often assayed as particulate preparations. Solubilization and purification of individual enzymes of the dolichol pathway have proven to be difficult and in most cases not particularly successful.

DOL Metabolism in Spermatogenic Cells

James and Kandutsch (1980c) studied DOL biosynthesis in X-irradiated or genetically mutated mice that were deficient

in spermatogenic cells. Testes from these mice, when compared to normal controls, demonstrated markedly reduced ratios of ^{14}C acetate incorporation into DOL as compared to cholesterol. These results suggested that the high rate of DOL synthesis in mouse testes may be attributed to one or more types of spermatogenic cells, although Sertoli cells may not be excluded. It was subsequently shown that prepuberal mouse pachytene spermatocytes incorporate acetate into DOL at a rate which is 5 times higher than that of leptotene and zygotene spermatocytes, and that a high rate of acetate incorporation into DOL is maintained in adult pachytene spermatocytes and round spermatids (Potter et al., 1981b).

A developmental study of DOL kinase activity in sexually maturing rats, 15-60 days of age, showed the appearance of detectable levels of activity at 21 days, a peak at 24 days and a subsequent decline to adult levels. The developmental pattern of this enzyme suggests its association with the later stages of spermatocyte development (Berkowitz & Nyquist, 1986).

Allen and Ward (1987) also reported changes in specific activity of DOL kinase during testicular development in the rat. They showed that the specific activity of kinase peaked at day 30, whereas the level of endogenous DOL P, as measured indirectly by the DOL P dependent mannosyl transferase activity, rose to a peak around day 15. Since the optimal activity of DOL kinase peaked later (day 30) than the peak in

DOL P levels (day 15), it was suggested that DOL kinase may function primarily in maintaining, adequate levels of DOL P for glycoprotein biosynthesis after the initial burst of DOL P biosynthesis. Therefore, DOL kinase was postulated to have little or no effect in regulating the rise in DOL P levels during the initial phases of differentiation during spermatogenesis. It was suggested instead that dehydro DOL PP synthase or DOL PP phosphatase may be the putative regulatory enzyme which directly affects the levels of DOL P in spermatogenic cells at different stages of development. The studies described here will extend our understanding of the regulation of DOL P metabolism in the testicular system.

Glycoproteins in Sertoli Cells

Sertoli cells are histologically and physiologically fundamental for spermatogenesis, since they are the only somatic epithelial component of the seminiferous tubules (Fawcett, 1975). The close physical association of Sertoli cells with the spermatogenic cell and the organization of this association into a cyclic pattern have been described in detail (Clermont & Perey, 1957). The characterization of Sertoli cells as nursing cells of testis was based originally on the morphological cellular relationship in the testis. The concept of Sertoli cells functioning as a support or regulatory factor has been confirmed by both biochemical and endocrine studies. It has been postulated that the

androgenic and tropic hormone action on spermatogenesis is mediated by the Sertoli cells. These cells have both FSH and androgen receptors (Sanborn et al., 1977; Sanborn et al., 1979; Means & Vaitukaitis, 1972) and show an appropriate temporal relationship between hormone binding and cell response. For example, there is nuclear accumulation of androgen and stimulation of RNA polymerase II activity in cultured Sertoli cells when FSH or testosterone were added in the media (Lamb et al., 1981). Therefore, it is possible to envision a scenario where the regulation of spermatogenesis is a result of the biochemical properties of Sertoli cells.

One of the possible mechanisms for Sertoli cells to influence spermatogenic cell development is via the secretion of proteins and glycoproteins which serve as signals or transport vehicles. The first glycoprotein to be identified as a Sertoli cell specific secretion product was androgen binding protein (French & Ritzen, 1973; Vernon et al., 1974). This protein is secreted by cultural Sertoli cells and its synthesis is regulated by FSH, testosterone, and vitamin A (Louis & Fritz, 1977; Karl & Griswold, 1980). The function of androgen binding protein is not clear but it is probably related to the capability of the protein to bind and transport androgens to the epididymis.

Sertoli cells synthesize and secrete a testicular transferrin. Skinner and Griswold (1982) speculate on the basis of biochemical and immunological similarities between

testicular and serum transferrin that testicular transferrin must play a role in the transport of iron from Sertoli cells to the spermatocytes and spermatids. More recently, Morales and Clermont (1986) have shown that during spermatogenesis Sertoli cells and spermatogonia internalized transferrin by receptor-mediated endocytosis at the base of the seminiferous epithelium.

Recent studies in the ram have shown that clusterin, a glycoprotein with a molecular weight of 37,000-40,000 is also synthesized de novo and secreted by Sertoli cells and transported to the rete testis (Rosenior et al., 1987).

Collectively, Sertoli cells actively secrete glycoproteins into the lumen of the seminiferous tubules and regulate spermatogenesis. Therefore, it is a relevant presumption that the synthesis of glycoproteins must be very active in these cells and that adequate DOL P must be provided by biosynthesis or uptake to support this function. The very high DOL content in Sertoli cell Golgi membrane suggests that Sertoli cells are actively involved in glycoprotein synthesis (Nyquist & Holt, 1986).

DOL in Sertoli Cells

Although a clear relationship between DOL P concentrations and the rate of glycoprotein synthesis has been established in many systems, the mechanism of regulation of glycoprotein biosynthesis in Sertoli cells is still

obscure. Furthermore, the question concerning the ability of the Sertoli cell to synthesize DOL was not addressed directly.

Nyquist and Holt (1986) recently measured the cellular and subcellular distribution of DOL in rat testis by a HPLC method and found that elutriation purified spermatogenic cells had very low concentrations of DOL. Pachytene spermatocyte and round spermatids contained 25.8 and 36.5 ng DOL/mg protein, respectively. Washed epididymal sperm also had a very low DOL content (18.8 ng DOL/mg protein). In contrast, the Sertoli cell enriched tubular fraction that was recovered during the preparation of purified spermatogenic cells showed the highest DOL content (3450 ng DOL/mg protein). These results implied that the Sertoli cells accumulate a major portion of the testicular DOL. This result at first appears inconsistent with the results of Potter et al. (1981b), who found low DOL synthesis in the testes from X-irradiated mice and spermatogenic deficient mice, which, although they were depleted of spermatogenic cells, were apparently normal with respect to the number and function of the Sertoli cells. Nyquist and Holt suggested in explanation that the bulk of testicular DOL may be synthesized in the spermatogenic cell and subsequently transported to the Sertoli cell. A possible route, they hypothesized, would be by Sertoli cell phagocytosis of residual body cytoplasm during spermiation.

The high content of DOL in the Sertoli cell may reflect a requirement for high DOL P to permit a rapid rate of glycoprotein biosynthesis during the spermatogenesis. It might be possible that Sertoli cell and spermatogenic cells may have a coordinate pattern of de novo DOL biosynthesis which is dependent on the presence of the other cell type. This would require that each cell type have the capacity to synthesize DOL without relying on intercellular DOL transport. In this way the Sertoli cell can independently regulate the level of DOL P and hence the synthesis and secretion of glycoproteins. Consequently, Sertoli cells can regulate spermatogenesis. Therefore, it was of interest to determine the capacity of Sertoli cells for de novo biosynthesis of DOL P in order to have a better understanding of the relationship between the Sertoli cells and spermatogenic cells during testicular development.

Significance

Dehydro DOL PP synthase is obviously a prime candidate as a regulated enzyme in the DOL biosynthesis and glycoprotein synthesis. It was of interest to determine if increased dehydro DOL PP synthase activity correlated with the increased rate of DOL synthesis in specific types of spermatogenic cells and the high DOL content in Sertoli cells, particularly since glycoprotein synthesis is active in

those cells. Recent data from our laboratory using DOL P dependent mannosyl transferase show that the level of DOL P increases dramatically from day 7 to day 20 in prepuberal rats (Allen & Ward, 1987). This time interval covers a period when the first group of spermatogenic cells are going through differentiation to become spermatids. It should be noted that the acrosomal enzymes, including the glycoprotein acrosin, are elaborated in early stages of spermatid formation. Several other proteins required for glycoprotein biosynthesis are also maximally expressed during this time interval, e.g. galactosyl transferase, N-acetylglucosaminyl transferase, and N-acetylglucosaminide fucosyltransferase in mice testes (Letts et al., 1974a). It is true for the androgen binding protein in rat Sertoli cell culture as well (Rich et al., 1983). Therefore, we have measured the specific activity of dehydro DOL PP synthase in spermatogenic cells and Sertoli cells during testicular development in order to determine its potential importance in the regulation of spermatogenesis.

A knowledge of changes in the concentration of intermediates and activities of enzymes in DOL metabolism is important if we are to understand the regulation of glycoprotein biosynthesis. Spermatogenesis offers a complex but good model system to study control mechanisms of DOL metabolism and DOL function in the biosynthesis of specific glycoproteins during cell differentiation. Information

gained from the assessment of dehydro DOL PP synthase activities in Sertoli cells and different types of spermatogenic cells may provide some insight into the mechanism of biochemical control of cell function during differentiation. This may ultimately be useful in the development of male contraceptives.

Objectives

The main objectives of this dissertation are to study dehydro DOL PP synthase and dehydro DOL PP phosphatase during spermatogenesis in rat.

Specific objectives are the following:

A. Optimize the assay conditions for dehydro DOL PP synthase.

B. Characterize the enzymatic products.

C. Determine the synthase specific activities in homogenate of testicular tubules of different aged rats.

D. Determine enzymatic activities in homogenate of different spermatogenic cell types (pachytene spermatocytes and spermatids), and Sertoli cells from rats testes.

Chapter II of this dissertation describes the study in achievement of objectives A through C. Chapter III describes the work in fulfillment of objective D.

CHAPTER II
DEVELOP AND OPTIMIZE AN ASSAY FOR DEHYDRO DOLICHYL DIPHOSPHATE SYNTHASE FROM RAT TESTES

Introduction

Elucidation of the mechanisms that regulate the synthesis of N-linked glycoproteins requires a clear understanding of the biosynthesis and metabolism of DOL and DOL P. For this reason we have undertaken studies on the biosynthesis and metabolism of dehydro DOL PP in rat testes. It seems that dehydro DOL PP is the precursor of DOL PP in the de novo biosynthesis pathway. Dehydro DOL PP synthase is responsible for the de novo biosynthesis of dehydro DOL PP from isopentenyl diphosphate and t,t-farnesyl diphosphate. This synthase has been demonstrated in several animal tissues (Grange & Adair, 1977; Wellner & Lucas, 1979; Wong & Lennarz, 1982; Adair & Keller, 1982; Adair et al., 1984; Adair & Cafmeyer, 1987a and 1987b; Baba et al., 1987), and is membrane associated. The products of this synthase were labile to acid and yielded petroleum ether soluble products indicating that the α-isoprene unit was unsaturated. Adair and Keller (1982) characterized the carbon number of the enzymatic product in rat liver and showed they are a group of

dehydro DOL Ps ranging in size from C_{75} to C_{95}. More recently, Baba et al. (1987) described the synthase in rat seminiferous tubules, but showed that the products of this enzyme were both dehydro DOL PP and dehydro DOL P. Hydrolysis of both of these products with a testicular phosphatase in the absence of NaF yielded the same chain length alcohols (C_{75}-C_{90}). The isolation of 2,3-dehydro DOL P and DOL P in some cases instead of the diphosphate derivatives undoubtedly reflects the action of a diphosphatase.

The methods for the assay of dehydro DOL PP synthase, which were described by Adair and collaborators (Adair & Keller, 1982; Adair et al., 1984), and Wellner and Lucas (1979) have been modified, as described below, to optimize the analysis of synthase activity in whole homogenates of small testicular samples obtained at different stages of spermatogenesis. In this assay, [^{14}C]-isopentenyl diphosphate and t,t-farnesyl diphosphate were chosen as substrates. As possible inhibitors of endogenous phosphatase, NaF and ATP were used to protect the substrates. Although Baba et al. (1987) showed that there were no major changes in the extent of formation of radiolabeled enzymatic products when NaF, $MgCl_2$ and ATP were omitted in the assay of partially purified subcellular membrane fraction, there were major changes in the nature of the <u>in vitro</u> products. For example, when Triton X-100 was omitted neither dehydro DOL PP

nor dehydro DOL P was formed, but a product, tentatively identified as presqualene monophosphate accumulated instead. Therefore, it was necessary to extend the earlier studies of Baba et al. (1987), to ensure that the assay developed for the synthase activity in crude tubular homogenates was measuring the desired activity.

This chapter 1) shows that treatment of the testicular homogenate by sonication yielded good dehydro DOL PP synthase activity while greatly reducing the formation of farnesol via another prenyl transferase activity; 2) describes the optimal parameters for the dehydro DOL PP synthase assay; 3) demonstrates unequivocally the precursor-product relationship between dehydro DOL PP and dehydro DOL P, and 4) elucidates a change in the enzymatic activity for dehydro DOL PP synthase in the seminiferous tubules during early stages of development. A possible role of the dehydro DOL PP synthase in regulating the biosynthesis of DOL is discussed. The two-fold increase in the specific activity of this synthase between day 7 and day 23 and a similar decrease in activity between day 23 and day 60 shown in this chapter provided the impetus to evaluate (in Chapter III) the enzyme specific activity in different cellular populations of rat testes.

Materials and Methods

Materials. Male Sprague-Dawley rats were purchased from local suppliers. t,t-Farnesyl diphosphate was prepared as previously described (Baba and Allen, 1978). [1-^{14}C]-Δ^3-Isopentenyl diphosphate and [^{32}P]-orthophosphoric acid (carrier free) in dilute HCl was purchased from Amersham/Searle Corp. Silica gel 60 F_{254} and Cellulose F_{254} on plastic sheets were products of E. Merck. All other reagents were obtained from standard commercial sources.

Solvent Systems. The following solvents were used for extraction or chromatography: Solvent A, $CHCl_3$-CH_3OH (2:1); Solvent B, $CHCl_3$-CH_3OH-H_2O (3:48:47); Solvent C, diisobutylketone-glacial acetic acid-H_2O (8:5:1); Solvent D, 2-propanol-acetonitrile-0.1 M ammonium bicarbonate (45:25:30); Solvent E, 1-propanol-concentrated ammonia-H_2O (6:3:1).

Animal grouping number required for analysis. In order to have sufficient data for statistical analysis in each experiment, at least two rats, but most often three or more rats were used for each age group tested (See Appendix A). The excised testes (six or more) from each age group were pooled together, then two or three aliquots of the mixed samples were taken for assay. The data presented in each table or figure were usually means determined by two or three similar experiments (e.g. Appendices B and C).

Preparation of homogenate. Male Sprague-Dawley rats were decapitated. The testes were removed and perfused with enriched Krebs-Ringer bicarbonate medium (EKRB) via the testicular vessels. This procedure effectively removed all blood cells from testes. The tunica albuginea was removed and seminiferous tubules were gently expressed. In the case of the younger animals (3 and 7 days old), the testes and the tunica albuginea were removed under dissecting microscope without perfusion.

The seminiferous tubules were weighed then suspended at a ratio of 1:2 (w/v) with ice cold buffer (20 mM Tris-HCl pH 7.5 and 1 mM EDTA). This mixture was sonicated in an ice bath in a Sonic dismembrator (model 300, Fisher) three times for 20 seconds with 20 seconds intervals without sonication. The pink milky homogenate was used as the enzyme source. Protein quantitation was made by a modified method of Lowry et al. (1951). Accurate protein quantitation was extremely important for determining enzyme specific activity. Therefore, samples were saponified in order to solubilize all proteins and each solubilized sample was assayed in duplicate or triplicate for protein.

Assay for Dehydro DOL PP Synthase. The assay method was developed as adapted from the method described by Grange and Adair (1977). The level of the enzyme was monitored by measuring the formation of polyprenyl products, dehydro DOL PP and dehydro DOL P. It was particularly important to

eliminate the unwanted products, and only isolate and quantitate dehydro DOL PP and dehydro DOL P. Optimal assay conditions were also established. The dependency of product formation on Triton X-100, protein, isopentenyl diphosphate, farnesyl diphosphate concentration and time were determined.

The standard assay of the enzyme was carried out by incubation of 100 mM Tris-HCl buffer pH 7.5, 10 mM $MgCl_2$, 0.5% Triton X-100, 250 μM t,t-farnesyl diphosphate, 1.6 mM ATP, 50 mM NaF, 36 μM [1-^{14}C]-isopentenyl diphosphate ($1.1x10^6$ dpm, 53 $\mu Ci/\mu$ mole), and 1.0 mg of enzyme protein in a final incubation volume of 0.25 ml for 1 hr at 37°C. The reaction was stopped by the addition of 0.25 ml of 1 M KOH. Then the mixture was heated at 100° C for 30 min to saponify the membrane bound lipids. Afterwards, the mixture was cooled in an ice bath, 0.25 ml of 1 M HCl and 1.25 ml of 2 M KCl were added. This mixture was extracted twice with 1 ml aliquots of Solvent A.

The combined solvent A extract was washed first with 2 ml of deionized water, then with 2 ml of Solvent B. When the extent of product formation was to be quantitated, a 1 ml aliquot was taken from the organic extract, dried in a scintillation vial and 10 ml of toluene based scintillation fluid (Scinti Verse II, Fisher) were added for analysis of the radioactivity. Radioactivity was then determined in a scintillation counter.

Thin Layer Chromatography (TLC) of Reaction Products. The remainder of extract (4.5 ml) was brought to dryness with a N_2 stream. Five drops of Solvent A were added to the dried residue and the tube was vortexed thoroughly. The resulting solution was applied to Silica gel 60 on plastic sheets which were previously cut into 4 cm x 20 cm sections. Five additional drops of Solvent A were used to wash the sample tube and this wash was added to the sample at the origin of the TLC sheets. The TLC sheets were then developed in a chamber with Solvent C.

The developed TLC sheets were subjected to either scanning with a radiochromatogram scanner (Packard model 7201) (Fig. 2-1-I) or autoradiography for 3-5 days on X-omat AR Kodak film (Fig. 2-1-II). The positions of migration of authentic DOL P standard and the radiolabeled products were correlated. Sections corresponding to the migration of dehydro DOL PP and dehydro DOL P were scraped into scintillation vials and 10 ml of scintillation fluid was added to each vial for radiochemical analysis. The level of activity was expressed in pmoles of [^{14}C]-isopentenyl diphosphate incorporated into dehydro DOL PP and dehydro DOL P /mg protein.

Preparation of Isopentenyl [α,β-^{32}P] Diphosphate. Isopentenyl [α,β-^{32}P] diphosphate was synthesized from 3-methyl-3-buten-1-ol and ^{32}Pi according to the procedure of Cramer and Bohm (1959). [^{32}P]-Orthophosphoric acid (0.5 mC$_i$,

carrier free) in dilute HCl was dried in the reaction vessel over P_2O_5 under N_2. Then 0.5 μmoles crystalline H_3PO_4, 6.25 μmoles triethylamine, 2 μmoles 3-methyl-3-buten-1-ol, and 12 μmoles trichloroacetonitrile in 60 μl acetonitrile were added and the reaction permitted to proceed for 5-7 hours at room temperature. The reaction was stopped by adding 200 μl of 10 mM NH_4OH. The reaction products was separated by TLC (Cellulose F_{254}) in Solvent D. The radioactive ^{32}P-band migrating beside authentic [^{14}C]-isopentenyl diphosphate (R_f=0.35) was scraped from the plate, packed in a glass column and [^{32}P]-isopentenyl diphosphate was eluted with 2 ml of methanol at room temperature. The nature of the ^{32}P-labeled product was verified by TLC along with authentic isopentenyl diphosphate by TLC on Silica 60 F_{254} in Solvent E.

Base Hydrolysis of Dehydro Dol PP. The putative dehydro DOL PP was biosynthesized from [^{14}C]-isopentenyl diphosphate and farnesyl diphosphate with a testicular homogenate according to the standard assay method. Enzymatic products were separated by TLC on Silica 60 F_{254} as already described. The dehydro Dol PP region on TLC (R_f=0.40) was localized by autoradiography and scraped into a conical test tube. The product bound to Silica gel was suspended in 1 ml of 3.5 M KOH in 70% methanol and the hydrolysis was carried out at 100° C for 2 hr. At the end of the hydrolysis, 2 ml of water was added to the mixture and the polyprenol

hydrolysis products were extracted with 2 ml of Solvent A. Then the lower phase was subjected to TLC analysis. The authentic markers, [^{14}C]-dehydro DOL P and non-radiolabeled DOL P were chromatographed in parallel to the hydrolysis products and the developed TLC sheet was subjected to autoradiography.

Results

Optimization of Synthase Reaction and Characterization of the Enzymatic Products. The typical prenyl transferase assay for the long chain polyprenyl diphosphate synthase in bacteria measures the amount of acid labile and organic solvent extractable product (Keenan & Allen, 1974). However, this method could not be satisfactorily applied for the quantitation of dehydro DOL PP synthase, because of the synthesis of other isoprenoid products with acid lability and extractablity similar to the dehydro DOL phosphates. A more accurate method was developed which involved 1) $CHCl_3$-CH_3OH extraction of the reaction products after saponification of the reaction components, 2) application of TLC to separate the dehydro DOL PP and dehydro DOL P from shorter chain polyprenyl phosphates and free polyprenols, and 3) determination of the sum of the dehydro DOL PP and dehydro DOL P formed. This method was satisfactorily applied to the identification of dehydro DOL PP synthase in the microsomal

fraction isolated from homogenates of seminiferous tubules from rat testes (Baba et al., 1987).

It was necessary to optimize the method to accurately assay the enzyme in homogenates of tubules taken from animals of different ages. Homogenates were prepared by the sonication of buffered suspensions of tissue instead of disruption with a glass homogenizer as previous described (Baba et al., 1987). In this study, sonication was found to be the only satisfactory procedure for disruption of the small amounts of tissue available from 3- and 7-day-old rats. Sonication also had the added advantage of denaturing the prenyl transferase, farnesyl diphosphate synthase, as exhibited by an elimination of radioactive product chromatographing with an Rf similar to exogenously added farnesol (Fig. 2-1-I, Panel B).

^{32}P and ^{14}C Ratios in the Mono and Diphosphate Products. The carbon chain lengths of the long chain polyprenyl products obtained by *in vitro* biosynthesis were previously shown to be the same (Baba et al., 1987). Since these two compounds had the same chain length, but they chromatographed on TLC and anionic exchange columns in a manner consistent with mono- and diphosphorylated products, they were assumed to be the dehydro Dol P and dehydro Dol PP. The ratios of phosphate to polyprenol chain were determined here for the putative dehydro Dol P and dehydro Dol PP in order to clarify this earlier assumption. Mixtures of [α,β-

^{32}P]-isopentenyl diphosphate and [^{14}C]-isopentenyl diphosphate were incubated with farnesyl diphosphate and tubular homogenates in the standard assay. The products were separated by TLC as usual and the ratios of radiolabel incorporated from [^{32}P]- and [^{14}C]-isopentenyl diphosphate were determined. Since the chain lengths of the polyprenol products have been established to be the same, the relative ratio of ^{32}P incorporated/^{14}C incorporated into the polyprenyl diphosphate is expected to be twice that ratio for the monophosphate. The results of such a test under two experimental incubation conditions are illustrated in Table 2-1. They support the hypothesis that the two products are the dehydro DOL P and dehydro DOL PP.

Several experiments were carried out to demonstrate the dependency of the enzyme activity on Triton X-100 concentrations and incubation times as well the extent and type of product formed. These experiments also serve to test the precursor-product relationship between the diphosphate and monophosphate.

Triton X-100 stimulated dehydro DOL PP synthase activity (Fig. 2-2, Panel C) with formation of dehydro DOL PP (Panel A) and dehydro DOL P (Panel B). The dependencies of the extent of mono- and diphosphate product formation on the concentration of detergent were quite different after incubation for 1 hr. Dehydro DOL PP formation was generally

Figure 2-1. Separation of Enzymatic Products by TLC.

(I) The products from the reaction of t,t-farnesyl diphosphate and [^{14}C]-isopentenyl diphosphate with homogenates of seminiferous tubules prepared with a glass-teflon homogenizer (Panel A) or by sonication (Panel B) were extracted with $CHCl_3/CH_3OH$ and subjected to TLC on silica gel sheets as described in the text. Arrows represent the position of migration of exogenous DOL P and farnesol.

(II) A example of autoradiography from enzyme assay.

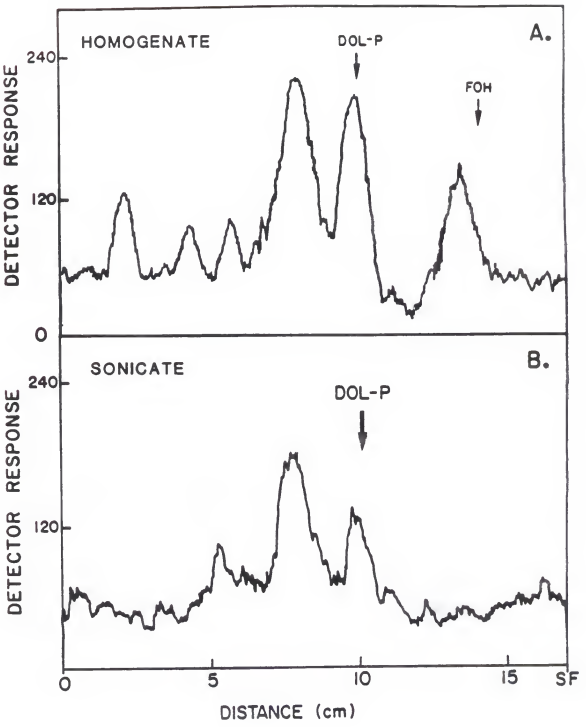

HOMOGENATE
DOL-P
FOH
A.
240
120
0
DETECTOR RESPONSE
SONICATE
DOL-P
B.
240
120
DETECTOR RESPONSE
0
5
10
15
SF
DISTANCE (cm)

Figure 2-1 (continued)

Table 2-1

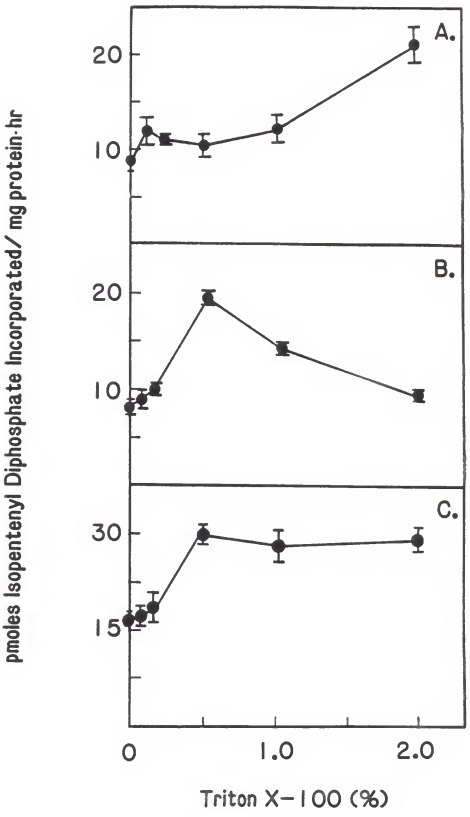
Incorporation of Δ^3-[1-^{14}C] Isopentenyl Diphosphate and [α,β-^{32}P]-Isopentenyl Diphosphate into Dehydro DOL PP and Dehydro DOL P[a]

Radiolabeled Substrate	Experiment 1 [^{32}P]-IPP 5 nmol (1.36 μCi)	[^{14}C]-IPP 4 nmol (0.21 μCi)	RATIO $\frac{^{32}P\text{cpm}}{^{14}C\text{cpm}}$	Experiment 2 [^{32}P]-IPP 10 nmol (2.72 μCi)	[^{14}C]-IPP 4 nmol (0.21 μCi)	RATIO $\frac{^{32}P\text{cpm}}{^{14}C\text{cpm}}$
Radiolabeled Product	(cpm incorporated)			(cpm incorporated)		
Dehydro DOL PP (A)	424	1489	0.28	1127	883	1.28
Dehydro DOL P (B)	217	1526	0.14	533	936	0.57
(A)/(B)			2.0			2.2

[a] The enzyme assay was carried out as described in the text. The reported values of cpm have been corrected for overlap of ^{32}P into the ^{14}C channel. (A)/(B) represents the relative ratio of radiolabel from ^{32}P incorporated into the diphosphate compared to the monophosphate.

Figure 2-2. Triton X-100 Dependency on the Formation of Dehydro DOL PP and Dehydro DOL P.

Incubations containing 100 mM Tris-HCl buffer (pH7.5), 10 mM $MgCl_2$, the indicated percentage of Triton X-100, 250 μM t,t-farnesyl diphosphate, 1.6 mM ATP, 50 mM NaF, 36 μM [1-^{14}C]-isopentenyl diphosphate, and 1.0 mg of enzyme protein in a final volume of 0.25 ml were carried out at 37° C for 60 minutes. The formation of [^{14}C]-dehydro DOL PP (Panel A) and [^{14}C]-dehydro DOL P (Panel B) were estimated by the method described before. Panel C represents dehydro DOL PP synthase activity (A+B).


A.
B.
C.
20
10
20
10
30
15
0
1.0
2.0
Triton X-100 (%)
pmoles Isopentenyl Diphosphate Incorporated/ mg protein-hr

stimulated with increasing detergent concentration throughout the concentration range shown in the figure, while dehydro DOL P formation was optimal at 0.5% Triton X-100. This suggests that when the Triton X-100 concentration was higher than 0.5%, a previously active dehydro DOL PP diphosphatase was inhibited. The sum of dehydro DOL PP and dehydro DOL P production was unchanged at Triton X-100 concentrations of 0.5% and higher.

The product ratio of dehydro DOL P to dehydro DOL PP shifted to favor the monophosphate when the detergent concentration in the incubation mixture was decreased midway through the incubation period. A part of the tubular homogenate was incubated at 37° C with substrates in either 0.5% Triton X-100 or 2% Triton X-100. In each case the products were analyzed after 1 hr and 2 hr. The incubation with 0.5% Triton X-100 gave dehydro DOL P as the predominant product at both 1 hr and 2 hr (Fig. 2-3, Panel A), whereas with 2% Triton X-100, the slower migrating product, dehydro DOL PP, was the predominant product at both time points (Fig. 2-3, Panel B). However, when a similar incubation was carried out in 2% Triton X-100 for the first hour to favor dehydro DOL PP formation and then the concentration of Triton X-100 changed to 0.5% during the second hour of incubation, the predominant product observed at the end of the second hour was dehydro DOL P (Fig. 2-3, Panel C).

Figure 2-3. Dependence of Product Formation on Triton X-100 Concentration and Incubation Time.

Sonicates of seminiferous tubules were assayed under standard conditions except that Triton X-100 and time of incubation were varied as shown. Dehydro DOL PP and dehydro DOL P were analyzed separately in reaction mixtures incubated for 1 hour and 2 hours in 0.5% Triton X-100 (Panel A), 2% Triton X-100 (Panel B) and from a reaction mixture which was incubated for 1 hour in 2% Triton X-100 and then diluted four fold with all reaction constituents except enzyme and Triton X-100 and incubated for an addition 1 hour (Panel C).

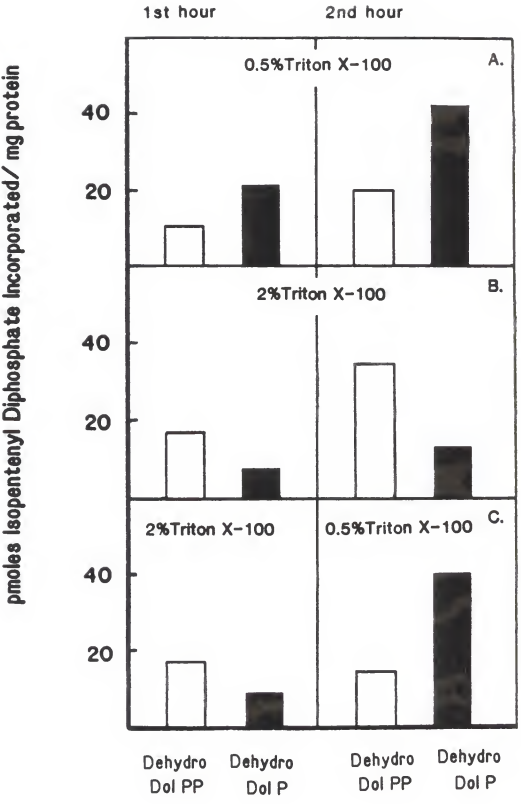

1st hour
2nd hour
0.5%Triton X-100
A.
40
20
2%Triton X-100
B.
40
20
2%Triton X-100
0.5%Triton X-100
C.
40
20
pmoles Isopentenyl Diphosphate Incorporated/ mg protein
Dehydro Dol PP
Dehydro Dol P
Dehydro Dol PP
Dehydro Dol P

Table 2-2

Formation of Enzymatic Product at Different Triton X-100 Concentrations in Pulse-Chase Experiment[a]

Time	Conditions	Isopentenyl Diphosphate Incorporated (pmoles) dehydro DOL PP	%	dehydro DOL P	%
1st hour	2% Triton	68	71	27	29
2nd hour	0.5% Triton	49	41	70	59

[a]Incubation conditions were the same as described in legend of Fig. 2-3 except that 4.81 mM unlabeled isopentenyl diphosphate was added to the reaction mixture after the first hour, reducing the specific activity of [^{14}C]-isopentenyl diphosphate 134-fold.

In all cases the sum of the two products increased with increasing time. This supports the presence of a diphosphatase which is inhibited by higher concentrations of Triton X-100.

The results of a similarly designed pulse-chase experiment support the same conclusion (Table 2-2). In this case non-radiolabeled isopentenyl diphosphate was added to the incubation mixture after one hour incubation in 2% Triton X-100. The reaction was continued for an another hour with addition of farnesyl diphosphate and enzyme but under lower Triton X-100 concentration (0.5%). Any polyprenyl phosphate made during the second hour of incubation, the chase phase, would not have been radiochemically detectable under the conditions used. Since there was a loss in radiolabeled product migrating as the putative dehydro DOL PP concomitant with an increase in radiolabeled dehydro DOL P, it can be concluded that the slower migrating product is a precursor of dehydro DOL P, and therefore it was dehydro DOL PP.

The precursor-product relationship between dehydro DOL PP and dehydro DOL P was also shown in a more detailed time dependent study illustrated in Fig. 2-4. The formation of the mono- and diphosphate was determined under standard assay conditions except that Triton X-100 concentration was raised to 1% to partially inhibit the diphosphatase activity. The distinct lag in the formation of dehydro DOL P relative to the diphosphate illustrates clearly the classical precursor-

product pattern. The formation of the two products increased linearly with time up to 60 min.

Base Hydrolysis of Dehydro DOL PP. The putative dehydro DOL PP was isolated by TLC and then subjected to saponification in 3.5 M KOH in 70% methanol for 2 hr at 100° C. Figure 2-5 shows that dehydro DOL P is the overwhelming product of base hydrolysis. This product is consistent with this enzymatic product being dehydro DOL PP.

Saponification in aqueous 0.1 M KOH at 100° C for 30 min is used in the standard assay. This procedure makes nonsaponifiable lipids more accessible for extraction and provides a better resolution of the enzymatic products on TLC, because of the hydrolysis and hence elimination of saponifiable lipids. Saponification under these milder conditions did not change the ratio between dehydro DOL PP and dehydro DOL P (results are not shown).

These results indubitably showed the *in vitro* reaction proceeds as follows: dehydro DOL PP synthase catalyzes the condensation of isopentenyl diphosphate and farnesyl diphosphate to generate dehydro DOL PP, which is the precursor of dehydro DOL P. Since the proportion of the diphosphate and monophosphate occasionally varied from one experiment to another, an accurate measurement of synthase activity required analysis of both products.

Figure 2-4. Time Course of Dehydro DOL PP and Dehydro DOL P Formation.

Sonicated seminiferous tubules were assayed under standard conditions except that 1.0% Triton X-100 was used and the time of incubation was varied as shown. The formation of dehydro DOL PP (solid triangles), dehydro DOL P (solid squares) and the sum of the both (solid circles), respectively.

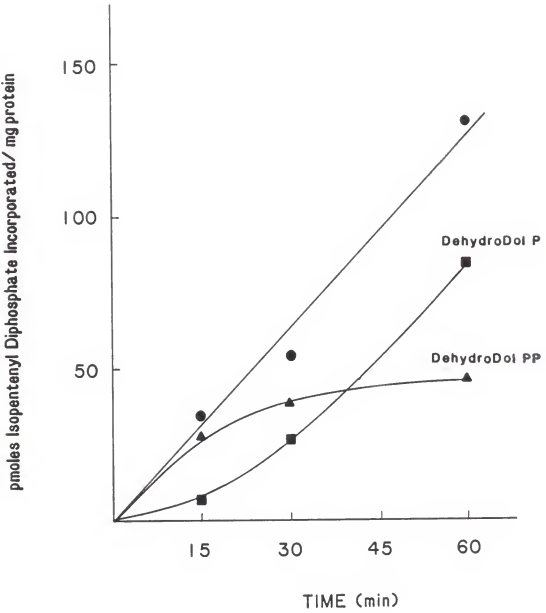

pmoles Isopentenyl Diphosphate Incorporated/ mg protein
150
100
50
15
30
45
60
TIME (min)
DehydroDol P
DehydroDol PP

Figure 2-5. Product of Base Hydrolysis of Dehydro DOL PP.

Dehydro DOL PP and dehydro DOL P were prepared by biosynthesis and isolated by TLC as described in the Methods. (A) Dehydro DOL PP was saponified. The hydrolysis product was extracted and chromatographed on Silica 60 F_{254} in Solvent C as described in the text. (B) as a control, dehydro DOL P was extracted from Silica 60 TLC sheets and chromatographed as described in A. The position of migration of authentic DOL P is shown by the arrow I. The position of dehydro DOL PP is shown by the arrow II.

F
I
II
O
A
B

Kinetics of the Enzyme. The enzyme assay conditions were optimized. Figure 2-6 shows the effect of varying protein concentration on the formation of the dehydro DOL PP (Panel A), dehydro DOL P (Panel B) and the sum of these two products (Panel C). These results indicated that the enzymatic activity increased linearly with protein concentration up to 2.4 mg protein incubated.

The dependency of dehydro DOL PP and dehydro DOL P formation on substrate concentration was studied. Figure 2-7 shows that the formation of dehydro DOL PP (Panel A), dehydro DOL P (Panel B), and the sum of these two products (Panel C) increased with increasing isopentenyl diphosphate concentration. A double-reciprocal plot of the sum of dehydro DOL PP and dehydro DOL P formation (Panel C) versus isopentenyl diphosphate concentrations showed (Figure 2-8) that the apparent Km=32μM and Vmax=1.23 pmoles/mg protein/min respectively.

Similarly, the dependency of the enzymatic products formation on the substrate farnesyl diphosphate was also studied. The experiment described (Figure 2-9) shows that farnesyl diphosphate was incorporated into dehydro DOL PP and dehydro DOL P. The formation of dehydro DOL PP (Panel A), dehydro DOL P (Panel B), and the sum of these two products (Panel C) increased with increasing concentration of farnesyl diphosphate. In Figure 2-10, a double-reciprocal plot of the sum of dehydro DOL PP and dehydro DOL P formation (Fig. 2-9,

Panel C) versus farnesyl diphosphate concentrations showed that the apparent Km=222μM and Vmax=0.67 pmoles/mg protein/min respectively. The Km values were higher than those observed for dehydro DOL PP synthase from Ehrlich ascites (Adair et al., 1984). This may reflect non-specific absorption of the substrates by other proteins in the crude homogenate and hydrolysis of the substrate by endogenous phosphatases, although ATP and NaF were included in the assay to minimize the action of phosphatases.

The time course of formation of dehydro DOL PP and dehydro DOL P in the standard assay is shown in Fig. 2-11. The formations of both dehydro DOL PP (Panel A) and dehydro DOL P (Panel B) are increased with increasing incubation time. Since the sum of these two products was linearly increased up to 60 minutes (Panel C), we chose one hour as the standard incubation time.

Changes of Dehydro DOL PP Synthase with age. The specific activity of dehydro DOL PP synthase in testicular homogenates of different aged rats was studied. The synthesis of dehydro DOL PP and dehydro DOL P from farnesyl diphosphate and [^{14}C]-isopentenyl diphosphate was determined in sonicates of tubules from rats aged 3-65 days. An example from several of these experiments is shown in Fig. 2-12. Under standard assay condition, the changes in the specific activity of the enzyme as measured by dehydro DOL PP

Figure 2-6. Effect of Protein Concentration on Dehydro DOL PP and Dehydro DOL P formation.

Incubations containing 100 mM Tris-HCl buffer (pH7.5), 10 mM $MgCl_2$, 0.5% Triton X-100, 250 μM t,t-farnesyl diphosphate, 1.6 mM ATP, 50 mM NaF, 36 μM [1-^{14}C]-isopentenyl diphosphate, and varying protein concentration in a final volume of 0.25 ml was carried out at 37° C for 60 minutes. The formation of [^{14}C]-dehydro DOL PP (Panel A) and [^{14}C]-dehydro DOL P (Panel B) were estimated by the method described before. Panel C represents dehydro DOL PP synthase activity (A+B).

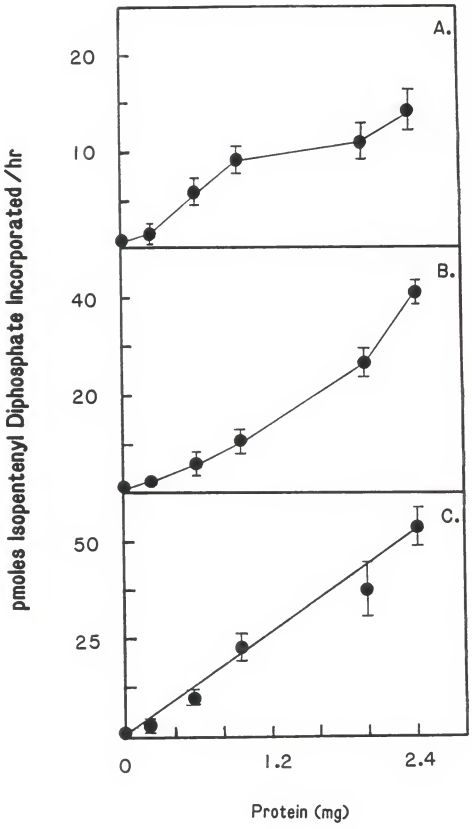

A.
B.
C.
20
10
40
20
50
25
0
1.2
2.4
pmoles Isopentenyl Diphosphate Incorporated /hr
Protein (mg)

Figure 2-7. Isopentenyl Diphosphate Concentration Dependency on the Formation of Dehydro DOL PP and Dehydro DOL P.

Incubations containing 100 mM Tris-HCl buffer (pH7.5), 10 mM $MgCl_2$, 0.5% Triton X-100, 250 μM t,t-farnesyl diphosphate, 1.6 mM ATP, 50 mM NaF, the indicated concentration of [1-^{14}C]-isopentenyl diphosphate, and 1.0 mg of enzyme protein in a total volume of 0.25 ml were carried out at 37° C for 60 minutes. The formation of [^{14}C]-dehydro DOL PP (Panel A) and [^{14}C]-dehydro DOL P (Panel B) were estimated by the method described before. Panel C represents dehydro DOL PP synthase activity (A+B).

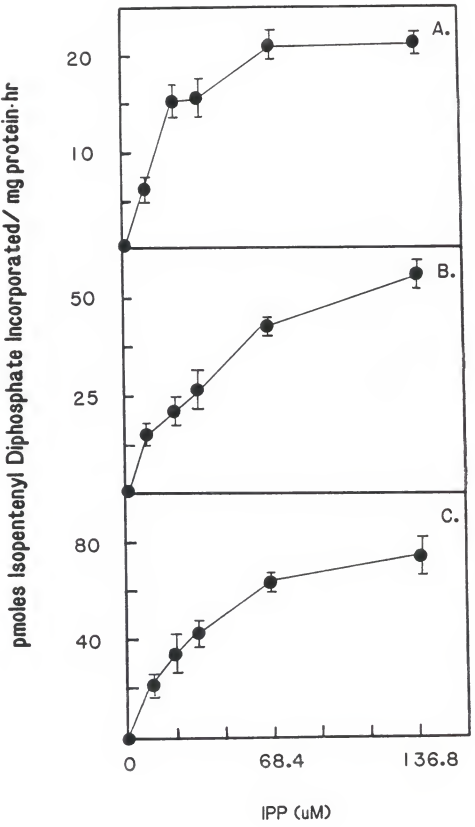

pmoles Isopentenyl Diphosphate Incorporated/ mg protein·hr
A.
20
10
B.
50
25
C.
80
40
0
68.4
136.8
IPP (uM)

Figure 2-8. A double reciprocal plot of the sum of dehydro DOL PP and dehydro DOL P formation (Fig. 2-7. Panel C) vs. isopentenyl diphosphate concentrations is presented.

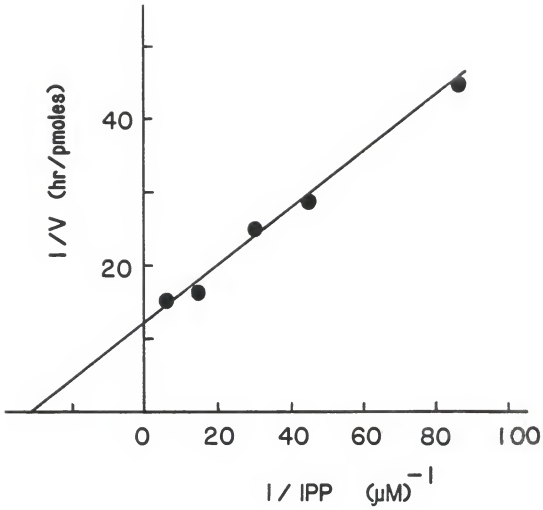

1/V (hr/pmoles)
40
20
0
20
40
60
80
100
1/IPP (μM)⁻¹

Figure 2-9. Farnesyl Diphosphate Concentration Dependency on the Formation of Dehydro DOL PP and Dehydro DOL P.

Incubations containing 100 mM Tris-HCl buffer (pH7.5), 10 mM $MgCl_2$, 0.5% Triton X-100, varying concentrations of t,t-farnesyl diphosphate, 1.6 mM ATP, 50 mM NaF, 36 μM [1-^{14}C]-isopentenyl diphosphate, and 1.0 mg of enzyme protein in a final volume of 0.25 ml were carried out at 37° C for 60 minutes. The formation of [^{14}C]-dehydro DOL PP (Panel A) and [^{14}C]-dehydro DOL P (Panel B) were estimated by the method described before. Panel C represents dehydro DOL PP synthase activity (A+B).

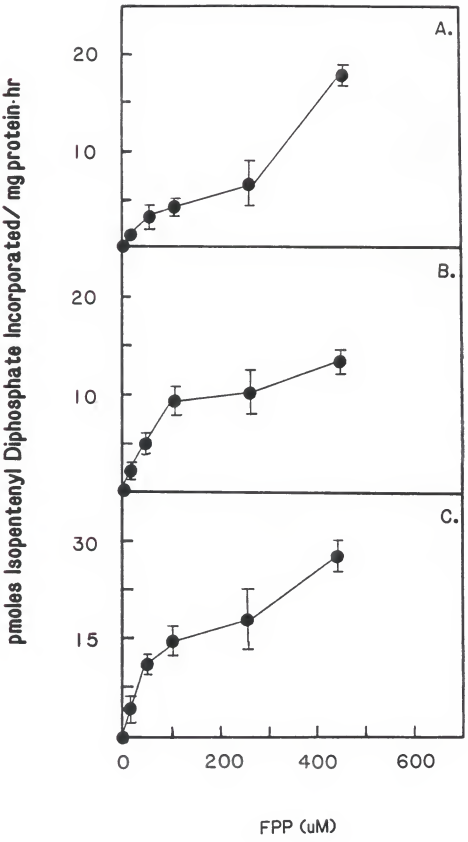

A.
B.
C.
20
10
20
10
30
15
0
200
400
600
pmoles Isopentenyl Diphosphate Incorporated/ mg protein·hr
FPP (uM)

Figure 2-10. A double reciprocal plot of the sum of dehydro DOL PP and dehydro DOL P formation (Fig. 2-9. Panel C) vs. farnesyl diphosphate concentration is presented.

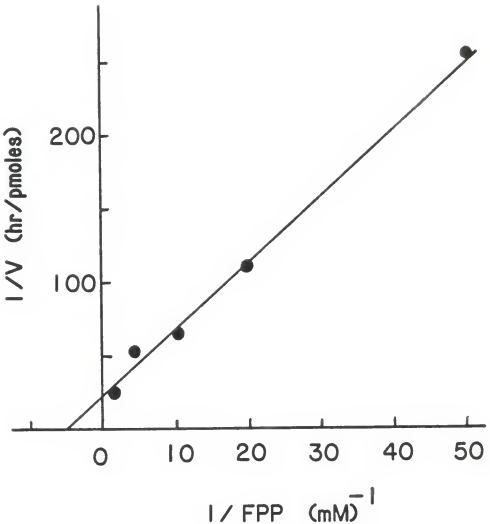

1/V (hr/pmoles)
200
100
0
10
20
30
40
50
1/FPP (mM)$^{-1}$

Figure 2-11. Time Course of Incorporation of [^{14}C]-Isopentenyl Diphosphate into Dehydro DOL PP and Dehydro DOL P.

Incubations containing 100 mM Tris-HCl buffer (pH7.5), 10 mM $MgCl_2$, 0.5% Triton X-100, 250 μM t,t-farnesyl diphosphate, 1.6 mM ATP, 50 mM NaF, 36 μM [1-^{14}C]-isopentenyl diphosphate, and 1.0 mg of sonicated of seminiferous tubules as enzyme protein in a total volume of 0.25 ml were carried out at 37° C for the indicated times. The formation of [^{14}C]-dehydro DOL PP (Panel A) and [^{14}C]-dehydro DOL P (Panel B) were estimated by the method described before. Panel C represents dehydro DOL PP synthase activity (A+B).

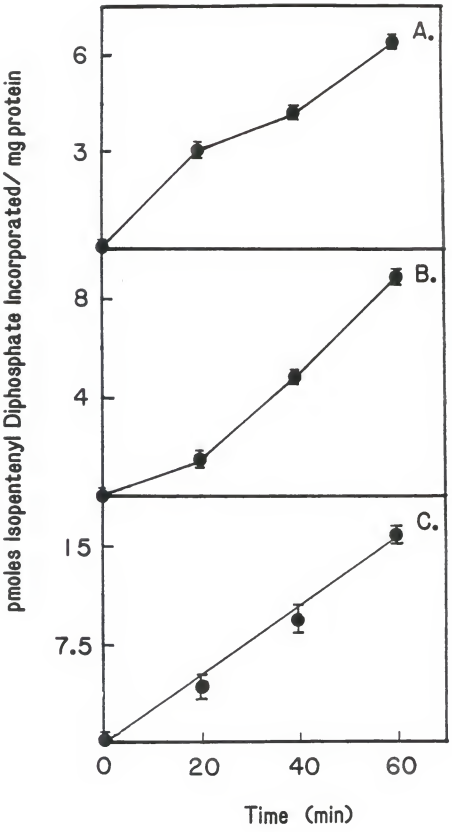

A.
B.
C.
6
3
8
4
15
7.5
0
20
40
60
Time (min)
pmoles Isopentenyl Diphosphate Incorporated/ mg protein

Figure 2-12. Dehydro DOL PP Synthase in Testicular Homogenate of Different Aged Rats.

Incubations containing 100 mM Tris-HCl buffer (pH7.5), 10 mM $MgCl_2$, 0.5% Triton X-100, 250 μM t,t-farnesyl diphosphate, 1.6 mM ATP, 50 mM NaF, 36 μM [1-^{14}C]-isopentenyl diphosphate, and 1.0 mg of enzyme protein from indicated aged rats in a final volume of 0.25 ml were carried out at 37° C for 60 minutes. The formation of [^{14}C]-dehydro DOL PP (Panel A) and [^{14}C]-dehydro DOL P (Panel B) were estimated by the method described before. Panel C represents dehydro DOL PP synthase activity (A+B).

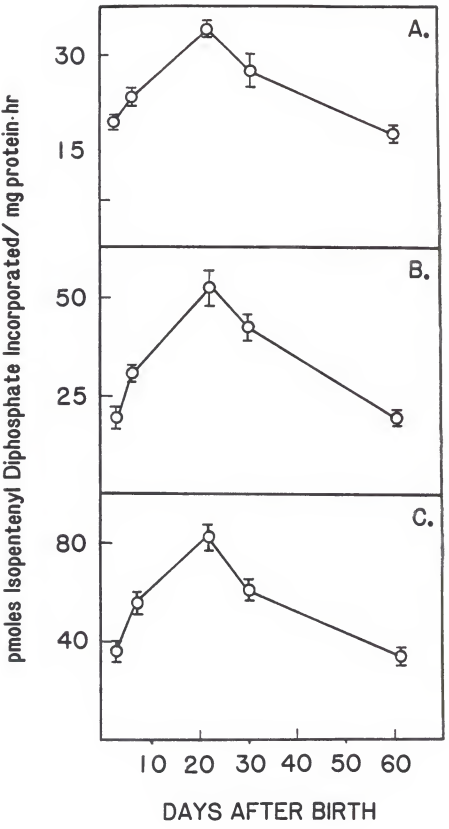
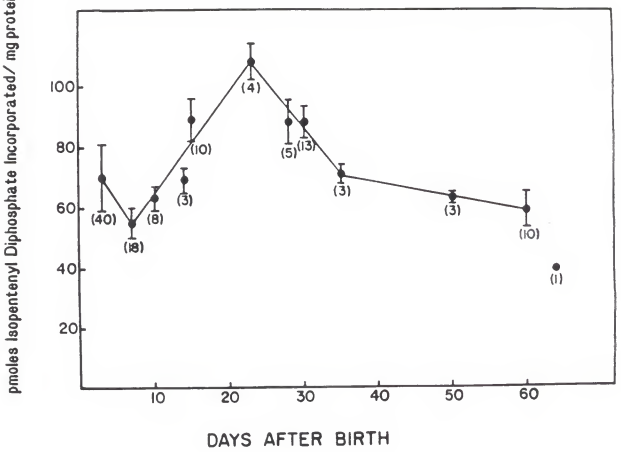

A.
30
15
B.
50
25
C.
80
40
10 20 30 40 50 60
DAYS AFTER BIRTH
pmoles Isopentenyl Diphosphate Incorporated/ mg protein·hr

Figure 2-13. Dehydro DOL PP Synthase Activity in Sonicates of Tubules from Rats of Different Ages.

The enzymatic activity was assayed under standard conditions with sonicates of seminiferous tubules as described in the Methods. The data is presented as the mean + standard deviation ($x \pm \frac{s_x}{\sqrt{n}}$). Numbers in parentheses indicate the number of animals used to prepare the tubules.


pmoles Isopentenyl Diphosphate Incorporated/ mg protein-hr
100
80
60
40
20
10
20
30
40
50
60
DAYS AFTER BIRTH
(40)
(18)
(8)
(3)
(10)
(4)
(5)
(13)
(3)
(3)
(10)
(1)

formation (Panel A) were somewhat smaller than changes in dehydro DOL P formation (Panel B) during testicular development. The sum of dehydro DOL PP and dehydro DOL P (Panel C) represents total specific activity of this enzyme. A composite of data from these studies using 118 rats is shown in Fig. 2-13. A two fold increase in tubular activity of the synthase occurred between day 7 and day 23 and a similar decrease in activity occurred between day 23 and day 60. A statistical treatment (Wilcoxon two-sample rank test) (Schefler, 1984) of these data shows a significant difference between 1) the 7-day-old and the-15-day old groups of rats ($p<0.005$) and, 2) the 30-day-old and the 60-day-old group of rats ($p<0.01$). Therefore, the peak of activity at 23 day old rats must be significantly higher than activity in rats aged both 7 days and 60 days.

Discussion

Early *in vivo* experiments have demonstrated that mevalonic acid serves as a precursor of dolichol in pig, rabbit and rat liver (Butterworth et al., 1966; Martin & Thorne, 1974). Using doubly and stereospecifically radiolabeled mevalonate and tissue slices, it was also shown that dolichol was synthesized from all-*trans*-farnesyl diphosphate by *cis*-addition of isoprene units (Gough & Hemming, 1970), suggesting that the biosynthetic pathway to

DOL branches from that to cholesterol at the level of farnesyl diphosphate. In vitro experiments with isopentenyl diphosphate as a precursor have shown that 2,3-dehydro DOL P, presumably one of the later intermediates in the DOL biosynthetic pathway, could be synthesized in preparations from hen oviduct (Grange & Adair, 1977), avian liver (Wellner & Lucas, 1979), Ehrlich tumor cells (Adair & Trepanier, 1980), mouse L-1210 cells (Adair & Cafmeyer, 1987a), yeast (Adair & Cafmeyer, 1987b), and testes (Baba et al., 1987). Testis has been shown to contain large quantities of dolichol (Rupar & Carroll, 1978; Tollbom & Dallner, 1986; James & Kandutsch, 1980c), and therefore it seems to be an appropriate tissue in which to investigate DOL biosynthesis.

Homogenates of rat seminiferous tubules have been previously shown to catalyze the synthesis of acid labile polyprenyl mono- and diphosphate (the α-unsaturated isoprene unit is acid labile). The enzymatic activity was dependent upon t,t-farnesyl diphosphate, isopentenyl diphosphate and divalent cation (Baba et al., 1987).

The conditions for the assay of dehydro DOL PP synthase in sonicates of rat seminiferous tubules have been systematically characterized and optimized here. The sonication of seminiferous tubules gives a few advantages for the assay. The sonication method, in contrast to homogenization with a glass homogenizer, can be easily applied to a small amount of testicular tissue, such as

obtained from 3- and 7-day-old rats. For instance, the pooled size of ten testes from 3-day-old rats is about the size of a rice grain (not a long grain!). Furthermore, sonication denatures the prenyl transferase, farnesyl diphosphate synthase, so that some of the side products from the assay are eliminated (Fig. 2-1-I). The reason for the loss in this prenyl transferase activity on sonication is not clear. Possibly, these enzymes are more sensitive to the heat generated by the sonication, despite cooling during this process.

It was necessary to optimize the conditions to accurately assay the specific enzymatic activity in sonicated seminiferous tissue from rats of different ages. Several pieces of evidence support the premise that the slow migrating TLC component (R_f=0.47) (Fig. 2-1), identified as dehydro DOL PP, was the initial enzymatic product, which was subsequently hydrolyzed <u>in vitro</u> to dehydro DOL P. Earlier work showed that the chain length of both the mono- and diphosphate derivatives of the polyprenyl product were the same (C_{75}-C_{90}) (Baba et al., 1987). The present study established more clearly the precursor-product relationship between the products dehydro DOL PP and dehydro DOL P. First, the sum of the mono- and diphosphorylated polyprenols increased linearly with a variety of increasing variables, i. e. Triton X-100 concentration (0 to 0.5% as shown in Fig. 2-2), protein (0 to 2.4 mg as shown in Fig. 2-6), time (0 to

60 min as shown in Fig. 2-11). Second, there is evidence for a detergent sensitive phosphatase that acts on the diphosphate to give the monophosphate. Third, kinetic experiments have shown a classical time dependent lag in monophosphate formation compared to diphosphate formation, whereas total phosphorylated polyprenol increased linearly. Fourth, direct chemical experiments were also performed to establish the precursor-product relationship between dehydro DOL PP and dehydro DOL P, such as double labeling experiment using [α,β-^{32}P]-isopentenyl diphosphate and [^{14}C]-isopentenyl diphosphate (Table 2-1), and base hydrolysis (Fig. 2-5).

The mild saponification used in each assay to release the membrane bound lipids did not change the ratio between dehydro DOL PP and dehydro DOL P. However, during a more vigorous saponification, dehydro DOL PP was hydrolyzed to dehydro DOL P as previous described (Adair and Cafmeyer, (1987). These experiments clearly showed that we can simply sum the radioactivity in the mono and diphosphate components as an accurate measurement of the total synthase activity.

Grange and Adair (1977) observed and therefore measured only dehydro DOL P formation in hen oviduct, although they commented that dehydro DOL P may be derived from dehydro DOL PP by the action of a phosphatase. In contrast, Adair and Cafmeyer (1987b) observed and measured only dehydro DOL PP formation in their studies in yeast. The current study showed that the testicular enzyme produces both dehydro DOL

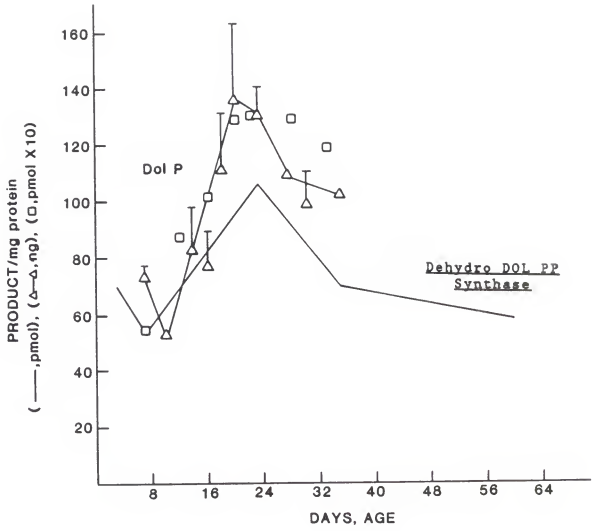
P and dehydro DOL PP, therefore, the determination of synthase activity required the measurement of both products, not dehydro DOL P or dehydro DOL PP alone.

DOL P is an indispensible carrier of oligosaccharides during glycoprotein biosynthesis, therefore, knowledge of its availability and the timing of its biosynthesis during early stages of differentiation may be important in understanding the regulation of spermatogenesis. This laboratory has shown that DOL P, as measured indirectly by a DOL P dependent mannosyl transferase assay, increased in immature rat testes about two fold between day 7 and day 30 after birth (Allen & Ward, 1987). Unpublished work of others in this laboratory has demonstrated similar results by direct measurement of DOL P with HPLC analysis of $CHCl_3/CH_3OH$ extracts of seminiferous tubules from different aged rats (Fig. 2-14).

Changes in dehydro DOL PP synthase during spermatogenesis in the rat have been studied here. The products of this synthase are undoubtedly intermediates in DOL P(P) biosynthesis. Dehydro DOL PP synthase was shown to increase two fold in specific activity between day 7 and day 23 after birth, and a similar decrease in activity between the day 23 and day 60. These findings parallel the changes in DOL P described above. Mechanisms which account for the increase in DOL P may include the phosphorylation of DOL with CTP dependent DOL kinase, de novo biosynthesis of DOL or as a result of release of DOL P from pools of DOL P and DOL PP

Figure 2-14. Comparison of Changes in DOL P Concentration, and Dehydro DOL PP Synthase Activity as a Function of Rat Age.

The specific activity of dehydro DOL PP synthase (——) are compared with the concentration of DOL P measured directly by HPLC (Δ) (unpublished observation, Allen, 1987) or by an indirect method (□), which was described in an earlier study (Allen & Ward, 1987).


PRODUCT/mg protein
(——,pmol), (Δ—Δ,ng), (□,pmol X10)
160
140
120
100
80
60
40
20
Dol P
Dehydro DOL PP
Synthase
8
16
24
32
40
48
56
64
DAYS, AGE

saccharides. Berkowitz and Nyguist (1986) showed a sharp rise in kinase activity at 21 days of age with a peak at 24 days. Allen and Ward (1987) showed a similar change in kinase activity, but the rise in activity appeared to be more gradual with a peak in activity at about 30 days and with one half maximal activity between day 20 and day 25. It seems unlikely that change in DOL kinase activity account for the changes in DOL P levels.

It has been suggested that alterations in the levels of the active, phosphorylated form of dolichol regulate the rate of N-linked glycoprotein synthesis (Lucas, 1979; Carson & Lennarz, 1979; Carson & Lennarz, 1981). The present results, which show that dehydro DOL PP synthase changes in specific activity at early stages of testicular development in rats (Fig. 2-13) suggest that increased DOL levels must accompany fluctuations in glycoprotein biosynthesis observed during this time period (Letts et al., 1978). The high rate of testicular DOL synthesis shown by Kandutsch and co-workers, as well as the temporal changes in DOL metabolism shown here during sperm differentiation, strongly suggest that membrane glycoproteins and alterations in the timing of their appearance may be significant regulators of spermatogenesis.

Potter et al. (1981b) showed a high rate of acetate incorporation into DOL in pachytene spermatocytes of adult mouse testes. At the same time, they also found increased HMG CoA reductase activity in these cells. Although HMG CoA

reductase may be one of the regulated enzymes in DOL synthesis (Rodwell et al., 1976; James & Kandutsch, 1979), the observed independent regulation of DOL and cholesterol biosynthesis can not be well explained by the control of this enzyme alone. A strong case is presented here for a regulatory role for dehydro DOL PP synthase during de novo DOL biosynthesis, because the specific activity of this enzyme rises in parallel with a two to three fold increase in DOL P concentration measured both directly and indirectly. Therefore, it can be concluded that dehydro DOL PP synthase is a regulatory enzyme responsible for controlling DOL biosynthesis on a pathway that is independent of cholesterol biosynthesis. This conclusion is consistent with the findings of Keller and Adair (1980), that DOL P synthase or long chain cis-prenyl transferase is a rate limiting factor in the biosynthesis of DOL P in liver. Recently, when radioactively labeled mevalonate was utilized to study in vivo and in vitro cholesterol, DOL and ubiquinone biosynthesis, considerable differences were observed between the rate of cholesterol synthesis and the rate of DOL and ubiquinone synthesis, while the rates of DOL and ubiquinone synthesis were quite similar (Elmberger et al., 1987). This observation suggested that the presence of important rate-limiting steps in the biosynthesis of DOL and cholesterol after mevalonate. This study suggests that dehydro DOL PP synthase may be one of these rate-limiting factors.

Since the cellular composition of the seminiferous tubules differs as a function of age during early stages of differentiation, changing cell populations may partially explain the difference in activity of dehydro DOL PP synthase in testes from rats of different ages. For instance, in 7-day-old rats the only spermatogenic cells are spermatogonia, by day 23, there are spermatogonia and pachytene spermatocytes as well, and after day 26, spermiogenic cells start appearing in the seminiferous tubules. It is likely that the different enzymatic activity observed during development can be accounted for by different cell populations with different enzymatic activity. The specific activity of different cellular populations are elaborated in the next chapter.

CHAPTER III
DEHYDRO DOLICHYL DIPHOSPHATE SYNTHASE ACTIVITY IN ENRICHED CELL POPULATIONS FROM RAT TESTIS

Introduction

Although DOL metabolism during testicular development has been the subject of several studies in recent years, a number of intriguing questions still remain unanswered. For example, do all rat spermatogenic cell populations have dehydro DOL PP synthase activity? Does each subpopulation of spermatogenic cells have the same enzymatic activity for the synthase? Is there a temporal relationship between synthase activity and developmental stage? Do Sertoli cells also have the synthase activity and synthesize its own dolichol? Is there a temporal relationship between spermatogenic cell and Sertoli cell synthase activity? Elucidating the answers to these questions will be of value in understanding the regulation in DOL metabolism as well as glycoprotein biosynthesis during spermatogenesis in rat. In this chapter, the specific activity of 2,3-dehydro DOL PP synthase in homogenates of protease treated seminiferous tubules, cell fractions enriched in spermatogenic cells or Sertoli cells from testis were measured as a function of the age of

prepuberal rats. The highest activity of this enzyme occurred in each case with cells from rats aged 23 days. Homogenates of cell fractions enriched in pachytene spermatocytes, spermatids or Sertoli cells were found to have higher synthase activity than a whole testicular homogenate or a mixture of cells prepared by protease treatment of tubules. The specific enzymatic activity in pachytene spermatocytes expressed per mg protein, was about 1.7 fold higher than in spermatids and about 8.3 fold higher than in spermatozoa. Therefore, the increase in spermatogenic cell synthase before day 23 can be accounted for by the appearance of the pachytene spermatocytes. Generally speaking, little net increase in enzyme occurred during or after meiotic cell division of spermatocytes into spermatids. Enzymatic activity decreased remarkably after the differentiation of spermatids into spermatozoa. Enzymatic activity in the enriched Sertoli cells was 1.5 to 1.7 fold higher than in the enriched spermatogenic cells between day 15 and day 30 of age. The increase in synthase specific activity in spermatogenic cells and Sertoli cells indicates that both are contributing to changes in the enzymatic activity in seminiferous tubules. This change may be important in regulating the availability of DOL P for glycoprotein synthesis during early stages of differentiation.

Spermatogenesis proceeds through a precise sequence of biochemical and morphological phases, spermatogonial, meiotic

and spermatid (Leblond & Clermont, 1952). During the second and third phases, many immunological and biochemical changes occur which are unique to the spermatogenic process. Among these changes are the elaboration of spermatogenic cell or Sertoli cell specific glycoproteins (Fenderson et al., 1984; Parvinen, 1982). For example, the spermatogenic cells produce an acrosomal protease precursor, proacrosin, and unique cell surface glycoproteins, which are required for fertilization. Proacrosin is first produced during the spermatid stage of differentiation but is retained throughout the remainder of spermatogenesis (Florke et al., 1983). Other proteins are only expressed in specific stages, so that they may be absent or low in the spermatogonial and spermatid phases but are abundant during the meiotic phase. (e.g. fucosyl transferase in prepuberal mouse testes) (Letts et al., 1974a).

The Sertoli cell, a non-germinal support cell of the seminiferous tubule, plays a critical role in the spermatogenic process by providing structural support, regulating spermatogenic cell movement, and compartmentation of spermatogenic cells from non-germinal cells, as well as mediating the movement of hormones, metabolites and nutrients to and from the developing spermatogenic cells (Ritzen et al., 1981). The Sertoli cell produces many glycoproteins that support these functions including androgen binding protein (Parvinen, 1982) and plasminogen activator (Lacroix

et al., 1977). Coordinated secretion of androgen binding protein by the Sertoli cell and the association of the Sertoli cell with pachytene spermatocytes during the meiotic phase even suggests the possibility of intercellular communication in regulating temporal expression of certain Sertoli cell proteins (LeMagueresse et al., 1980; Ritzen et al., 1982; Galdieri, 1984; LeMagueresse & Jegou, 1986).

The biosynthesis of these glycoproteins at specific phases during testicular development requires a coordinate functioning of a series of enzymes, so that cofactors and associated biochemical apparatus must be present in the cell at or before the time of glycoprotein expression or function. However, only a few studies have described the regulation of expression of these components as possible controlling factors of spermatogenesis.

Three of the glycosyl transferases, which are needed for the terminal reactions in glycoprotein oligosaccharide biosynthesis in rat and mouse testis, have been shown to increase in specific activity in a sequential manner, that parallels their use in the last steps of oligosaccharide processing (Letts et al., 1974a).

The availability of DOL P, another critical component in the synthesis of the N-linked glycoproteins, has been suggested as a rate-limiting factor in some developmental processes (Lucas, 1979; Harford & Waechter, 1980; Rossignol et al., 1980). Therefore, the study of DOL metabolism will

be undoubtedly useful for the research of glycoprotein regulation. James and Kandutsch (1980c) reported that mouse spermatogenic cells were more active than liver cells in DOL biosynthesis. Furthermore, Potter et al. (1981b) identified the pachytene spermatocytes as one of the most active spermatogenic cells in DOL synthesis. This prompted our study of dehydro DOL PP synthase, an enzyme that could contribute to increased DOL levels during spermatogenesis.

Chapter II described In vitro assays for the synthase, which were developed to measure changes in the potential of seminiferous tubules to biosynthesize dehydro DOL PP, a probable precursor in the biosynthesis of DOL P and DOL. The temporal expression of synthase correlated well with the increase of DOL P measured by HPLC methods in seminiferous tubules during early stages of differentiation in prepuberal rats. It was proposed, therefore, that the level of DOL P in rat seminiferous tubules might be controlled by the regulation of de novo dehydro DOL PP biosynthesis.

In the previous chapter, the specific activity of the dehydro DOL PP synthase was shown to fluctuate during testicular development; it was hypothesized that a difference in enzymatic activities during development might due to the presence of different cell populations with different enzymatic activities in rat testes. In this chapter, the methods of cell fractionation are described, the specific activities of the dehydro DOL PP synthase are measured in

different purified spermatogenic cell populations and in Sertoli cells. A model is presented which explains the time dependent change of the dehydro DOL PP synthase specific activity in tubules of prepuberal rats.

Materials and Methods

Materials. Sprague-Dawley rats were obtained from local suppliers. t,t-Farnesyl diphosphate was prepared as previously described (Baba & Allen, 1978). [^{14}C]-Δ^3-Isopentenyl diphosphate was purchased from Amersham/Searle Corp. Bovine serum albumin (BSA), trypsin, trypsin inhibitor, deoxyribonuclease, and collagenase were purchased from Sigma Chemical Corp. All other chemicals were of reagent grade.

Solutions. Phosphate buffered saline, essential amino acids (BME 50X) and MEM nonessential amino acids were obtained from Gibco Labs. Enriched Krebs-Ringer bicarbonate medium (EKRB) contained 120.1 mM NaCl, 4.8 mM KCl, 25.2 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4 \cdot 7H_2O$, 1.3 mM $CaCl_2$, and was enriched by the addition of 11 mM glucose, 1 mM glutamine, 10 ml/liter of essential amino acids, and 10 ml/liter nonessential amino acids. Streptomycin sulfate (100 μg/ml) and penicillin G (K^+ salt) (60 μg/ml) were also added to the medium. The solution was prepared from a stock solution immediately prior to use, filtered (0.30 μ

Millipore), and the pH adjusted to 7.3 by a 15-20 min aeration with 5% CO_2 in air. Glassware and other equipment was siliconized before use in order to reduce damage and adhesion of cells.

Preparation of cell suspensions. Rats aged 7-65 days were sacrificed and testes were removed as described in Chapter II. Testicular cell suspensions were prepared by a modification of the two-step enzymatic method described for mouse (Romrell, 1979). The decapsulated testes were placed in a 50-ml Erlenmeyer flask containing 20 ml of collagenase (1 mg/ml) and DNAse (1 μg/ml) in EKRB. The testes were incubated at 33°C in a shaking water bath operated at 120 cycles/min, until the seminiferous tubules were freely dispersed in the incubation medium (10-15 min). The dispersed seminiferous tubules were allowed to sediment and the supernatant was decanted. The isolated tubules were washed twice with EKRB. Then fresh EKRB (20 ml) containing trypsin (2.5 mg/ml) and DNAse (1 μg/ml) was added to the tubules and this suspension was incubated for 15 min in a shaking water bath as just described. The resulting cell suspension was gently pipetted approximately 50 times with a Pasteur pipet. Trypsin inhibitor (2.5 mg/ml) was added, and then the cell suspension was mixed with 10 ml of 0.5% BSA in EKRB and centrifuged at 200 x g for 10 min. The resulting pellet was washed three times with EKRB containing 0.5% BSA and 1 μg/ml DNAse and resuspended in the same solution after

the washing. The suspension was filtered through a nylon mesh (135 μ) to remove cell aggregates. The cell concentration was determined using a hemocytometer. This enriched spermatogenic cell suspension was finally suspended in EKRB containing 0.5% BSA and adjusted to a concentration of 2 x 10^6 cells/ml.

Spermatogenic Cell Fractionation. The entire cell separation procedure was carried out at 5° C. Spermatogenic cells were separated by a modification of the STA-PUT unit gravity procedure (Romrell et al., 1977). The sedimentation chamber was initially filled with 70 ml EKRB. Then the cell suspension, which contained 10^8 cells in 50 ml of 0.5% BSA in EKRB, was introduced into the chamber at a flow rate of 10 ml/min. The sample was followed by a linear gradient of 2% to 4% BSA in EKRB generated from two interconnected reservoirs, that contained 1100 ml of 4% BSA and 1100 ml of 2% BSA, respectively (total volume 2200 ml). Five min after loading the cell suspension, the flow rate was increased to 40 ml/min. Eighty minutes after loading the cell suspension, the chamber was drained in 10-ml fractions at a rate of 10 ml/min. Cell collection was finished within 5 hr after introducing the cell suspension to the chamber. The separated cell fractions were numbered and centrifuged at 200 g for 10 min; the supernatant were decanted; the resulting pellets were resuspended in 0.5 ml of EKRB. Aliquots were taken from individual samples and checked for cell type and

purity. The cells were examined by Nomarski differential interference and phase microscopy. The samples of enriched early spermatids (stages 1 through 8) and pachytene spermatocytes were pooled separately, washed three times with phosphate buffered saline solution and used immediately for the measurement of the enzymatic activity. The cellular purity of the pachytene spermatocyte (Fig. 3-1-A) was 70%; the major contaminants being Sertoli cells and spermatids. The purity of the spermatid (Fig. 3-1-B) fractions was 80%, with pachytene spermatocytes, the primary contamination.

Spermatozoa were obtained from adult rats (3 months of age). The cauda epididymis was removed and flushed with 1 ml of phosphate buffered saline via the ductus deferens. The collected spermatozoa were then washed three times in phosphate buffered saline and used for the enzymatic assay.

Sertoli Cell Preparation. Sertoli cells were prepared by modification of the procedure of Dorrington et al. (1975). Sertoli cells were isolated from Sprague-Dawley rats of specific ages. Tubules were treated with proteases as described above except that phosphate buffered saline was used in place of EKRB and more (3 μg/ml) DNAse was used. The process was similar to that for spermatogenic cell fractionation, only the Sertoli cells retained on the nylon filter were collected as the fraction enriched in Sertoli cells. These aggregates of 10-50 Sertoli cell was further treated for 3-4 min with a hypotonic solution of two-fold

diluted phosphate buffered saline to remove spermatogenic cells. Under these conditions the spermatogenic cells are lysed but the Sertoli cells retain their integrity. The cells were examined by Nomarski differential interference and phase microscopy to check for purity and integrity. The resulting cell suspension contained more than 80% Sertoli cells with a contamination of pachytene spermatocytes and spermatids (Fig. 3-1-C).

Homogenate Preparation. Homogenates were prepared from seminiferous tubules, cell suspensions from tubules, enriched spermatogenic cell populations and Sertoli cells. The excised testicular tubules were weighed, suspended at a ratio of 1:2 (w/v) in ice cold buffer (20 mM Tris-HCl pH 7.5 and 1 mM EDTA) and sonicated as described in Chapter II. Homogenates of various mixed and enriched cell fractions, were prepared similarly, except that in these cases the packed cells were suspended in two volumes of buffer (v/v). Protein quantitation was determined by the method of Lowry et al. (1951) before assay[1].

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[1]The cellular dissociation procedure used a relatively large amount of BSA (2-4%). There was a concern that binding of BSA to the purified cell fractions might lead to some error in protein determination of these isolated cells. Therefore, it was useful to estimate the binding of BSA to the enriched germ cells. Measurement of binding was carried out with [^{125}I]-BSA as a probe in 2% BSA. Protease dissociated germ cells were incubated with [^{125}I]-BSA for 3 min at room temperature then washed. The radioactivity on the cell in the beginning and the end of the test was compared. The results showed negligible non-specific binding of BSA to the cell surface (144 μg/14350 μg which is less than 1%) after the STA-PUT fractionation and the buffer

Dehydro DOL PP Synthase Assay. The enzyme activity was determined by the same assay method described in chapter II. The enzyme protein (1 mg) from homogenates of enriched cell populations or various cell mixtures were assayed as indicated. The products, dehydro DOL PP and dehydro DOL P were extracted with $CHCl_3/CH_3OH$ (2:1), isolated by TLC and quantitated as described in Chapter II. The level of enzymatic activity was expressed as the sum of the pmoles of isopentenyl diphosphate incorporated/mg protein. The relationship of these two products were extensively discussed in the previous chapter.

Results

Synthase Activity in Protease Treated Seminiferous Tubules. The synthesis of dehydro DOL PP and dehydro DOL P from farnesyl diphosphate and [^{14}C]-isopentenyl diphosphate was compared in sonicates of protease-treated and untreated tubules from rats aged 7-65 days (Fig. 3-2). It is necessary to determine if the enzyme in mixed tubular cell populations has the same enzymatic properties as that in the isolated tubules. In another words, did proteases treatment change the enzymatic properties of the separated tubular cells? The fluctuation in enzyme specific activities in the protease

washes. Therefore, the enzymatic activities measured in the study do not need to be corrected.

treated tubules was parallel to that seen in the untreated tubules. Although there was a decrease in specific activity in the protease treated tubules at all ages, there was no apparent selective loss of activity at any particular age[2].

Synthase Activity in Enriched Cells from Testis. The level of synthase activity was also evaluated in isolated spermatogenic cells and epididymal spermatozoa. The time course of formation of dehydro DOL PP and dehydro DOL P was evaluated in the enriched pachytene spermatocytes, spermatids and Sertoli cell (Figure. 3-3). The results indicated that the sum of product formation with 1 mg of protein from homogenates of each cell type increased linearly with increasing incubation time as shown in the chapter II for the tubular homogenates. Protease treatment did not have an adverse effect on the linearity of time dependent assay. The enzyme specific activity in Sertoli cell was the highest. Homogenates of cell fractions highly enriched for pachytene spermatocytes (70% purity) and spermatids (80% purity) had synthase activity equal to or higher than a whole testicular

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[2]The methodology for dissociating cells from seminiferous tubules was satisfactory, since 80-90% of the enzymatic activity still remained after the proteases treatment. Dispase, a commercial preparation of collagenase has also been reported to dissociate several tissues successfully. Therefore, dispase was tested as a replacement for collagenase in the cellular dissociation from seminiferous tubules. About 40% of the enzymatic activity in the collected dissociated cells was lost by this treatment (results are not shown).

Figure 3-1. Purity of Enriched Cell Fractions.

The testicular tubules were dissociated with proteases and the spermatogenic and Sertoli cells were separated as described in the Materials and Methods. The purity of each cell fraction, as estimated by Nomarski differential interference microscopy (x 600), was estimated to be 80% for spermatids (Panel A), 70% for pachytene spermatocytes (Panel B) and 70% for Sertoli cells (Panel C).

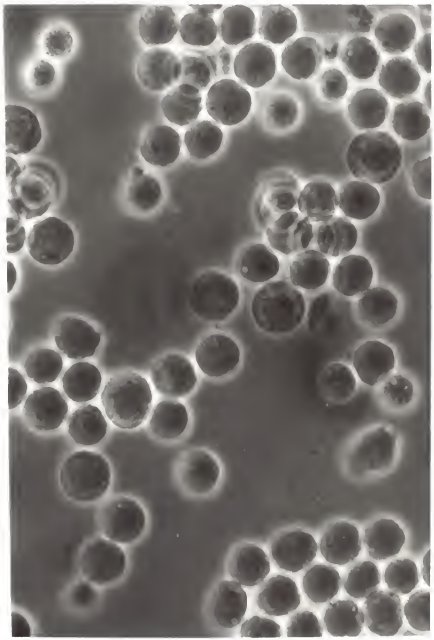


Figure 3-1. Panel A. Spermatids.

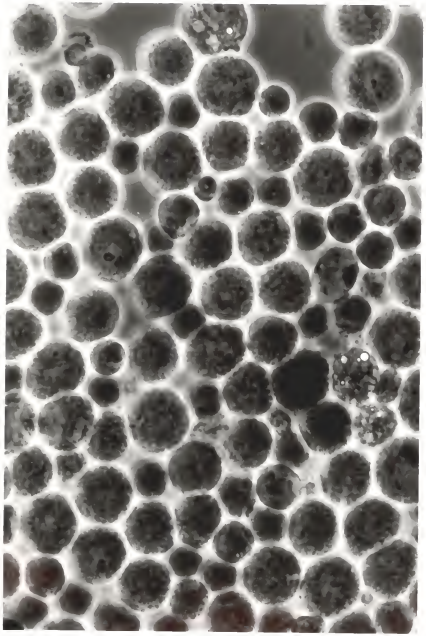


Figure 3-1. Panel B. Pachytene Spermatocytes (continued).

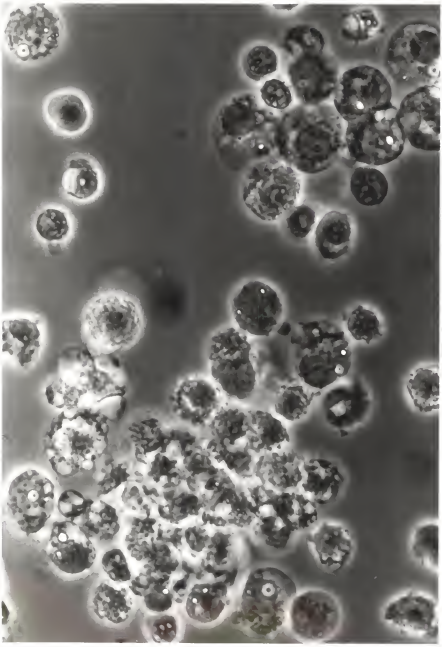


Figure 3-1. Panel C. Sertoli Cells (continued).

Figure 3-2. Dehydro Dolichyl Diphosphate Synthase Activity in Sonicates of Tubules from Rats of Different Ages.

The enzymatic activity was assayed under standard conditions with sonicates of seminiferous tubules treated (o) and untreated (•) with protease as described in the Materials and Methods. The data is presented as the mean ± standard deviation ($x \pm \frac{s_x}{\sqrt{n}}$). Numbers in parentheses indicate the number of animals used to prepare the tubules.

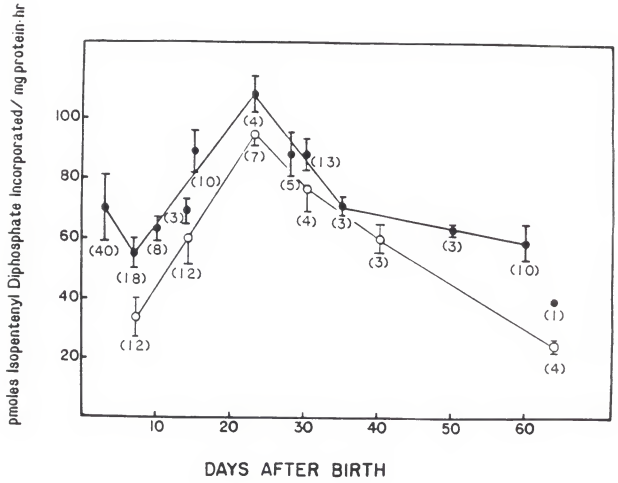
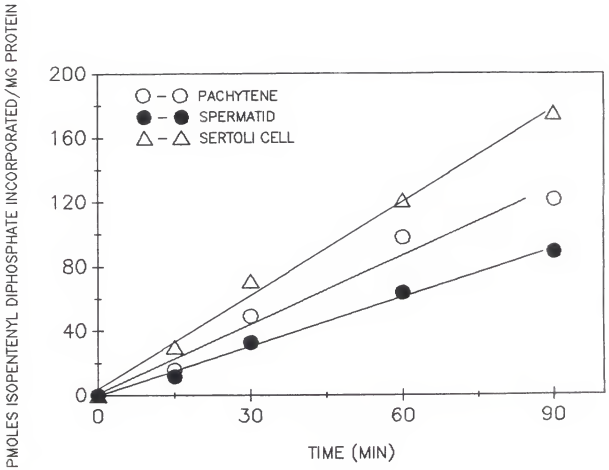

pmoles Isopentenyl Diphosphate Incorporated/ mg protein-hr
100
80
60
40
20
10
20
30
40
50
60
DAYS AFTER BIRTH
(40)
(18)
(8)
(3)
(10)
(12)
(4)
(7)
(5)
(4)
(13)
(3)
(3)
(3)
(10)
(1)
(12)
(4)

Figure 3-3. Time Course of Incorporation of [^{14}C]-Isopentenyl Diphosphate into Dehydro DOL PP and Dehydro DOL p.

Incubations containing 100 mM Tris-HCl buffer (pH7.5), 10 mM $MgCl_2$, 0.5% Triton X-100, 250 μM t,t-farnesyl diphosphate, 1.6 mM ATP, 50 mM NaF, 36 μM [1-^{14}C]-isopentenyl diphosphate, and 1.0 mg protein of sonicated of enriched pachytene spermatocyte, spermatid, and Sertoli cell from seminiferous tubules as enzyme protein in a total volume of 0.25 ml were carried out at 37° C for the indicated times. The sum of [^{14}C]-dehydro DOL PP and [^{14}C]-dehydro DOL P were estimated by the method described before. Sertoli cells are isolated from 23 day old rats, spermatogenic cells are from 40 day old rats.


PMOLES ISOPENTENYL DIPHOSPHATE INCORPORATED/MG PROTEIN
200
160
120
80
40
0
O – O PACHYTENE
● – ● SPERMATID
△ – △ SERTOLI CELL
0
30
60
90
TIME (MIN)

homogenate with or without protease treatment (Fig. 3-4). On the other hand, the spermatozoa and spermatogonial enriched cell fractions had activities less than 20% of the spermatocyte activity. When the enzymatic activity was expressed as pmoles/mg protein/hr, pachytene spermatocytes had an activity 1.6 fold higher than that seen with a mixture of cells obtained from protease treated seminiferous tubules. The enzymatic activity of the enriched spermatocytes was about 1.4 fold higher than enriched spermatids, 4.8 fold higher than spermatogonia and about 7.6 fold higher than spermatozoa. The enzymatic activity of the spermatogenic cells may also be expressed as pmoles product formed/10^6 cells/hr (Table 3-1). In this case the enzymatic activity of spermatocytes (28.9 pmoles/10^6 cells/hr) was 4.5 fold higher than that of spermatids (6.5 pmoles/10^6 cells/hr) and about 126 fold higher than that of spermatozoa (0.23 pmoles/10^6 cells/hr).

Synthase Activity in Sertoli Cells. Synthase specific activities were also measured in homogenates of Sertoli cells and a mixed spermatogenic cell population from rats 7-65 days old (Fig. 3-5). The fluctuations in the enzyme specific activities for both of these cell populations were parallel to that seen with the protease and non-protease treated tubules. In each case, the activities peaked at day 23. The enriched Sertoli cell specific activity ranged from 1.5-2.3

fold higher than that of the mixed spermatogenic cell population between day 14 and day 30.

Estimated Activities in Pure Cell Populations. Synthase specific activities in "pure" populations of the different spermatogenic cells and the Sertoli cells were estimated from the known purity of the enriched cell fractions and their specific activities. Table 3-2 shows that, after a correction was made for the contamination of each cell fraction for other spermatogenic cells, the enzyme specific activity in pachytene spermatocytes was 1.7, 5.3 and 8.3 fold higher than in spermatids, spermatogonia and spermatozoa, respectively. Estimates of the specific activities of "pure" Sertoli cells isolated from rats of different ages is shown in Table 3-3. There was a 4.5 fold increase in the enzyme activity of the Sertoli cell between day 7 and day 23 and 4.3 fold decrease in activity from day 23 to day 65.

In summary: 1) dehydro DOL PP synthase activity in spermatogenic cells and Sertoli cells peaks in rats at age 23 days, 2) the specific activity of the enriched and pure spermatogenic cell populations tested decreased in the following order: pachytene spermatocyte > spermatids > spermatogonia > sperm and 3) synthase specific activity for Sertoli cells was 1.5-1.7 fold higher than that of spermatogenic cells in rats between day 15 and day 30 of age.

Figure 3-4. Dehydro Dolichyl Diphosphate Synthase Activity in Enriched Spermatogenic Cell Population.

Synthase activity was measured in sonicates of seminiferous tubules treated and untreated with protease, spermatogonia enriched spermatogenic cells from tubules of 7 day old rat, pachytene spermatocytes, spermatids (from 40 day old rats) and epididymal spermatozoa (from 3 months old rats). The specific activity of the enzyme is presented as the sum of pmoles of dehydro DOL PP and dehydro DOL P formed/mg protein hr.

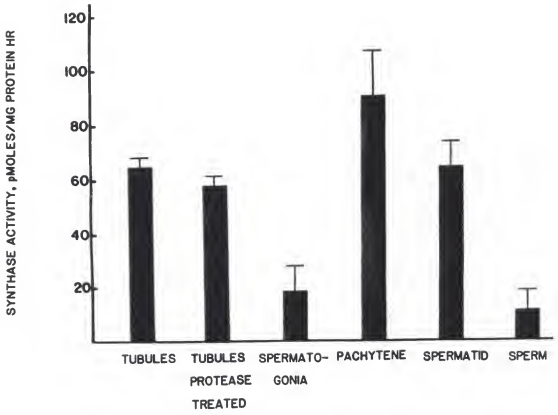

SYNTHASE ACTIVITY, pMOLES/MG PROTEIN HR
120
100
80
60
40
20
TUBULES
TUBULES PROTEASE TREATED
SPERMATO-GONIA
PACHYTENE
SPERMATID
SPERM

Table 3-1

Dehydro Dolichyl Diphosphate Synthase Activity in Enriched Spermatogenic Cells[a]

Experiment	Activity		Ratio
	pmoles/10^6 Cell · hr		
	Pachytene (P)	Spermatid (S)	P/S
1	34.9	7.9	4.4
2	33.1	7.9	4.1
3	22.9	5.9	3.9
4	25.0	4.5	5.6
Average	28.9 ± 2.9	6.5 ± 0.8	4.5 ± 0.4

[a]Enzyme activity was measured in cellular sonicates as described in the Materials and Methods and expressed as pmoles of isopentenyl diphosphate incorporated per 10^6 cell per hour. The cell numbers were calculated by using the conversion factors 258 μg protein = 10^6 cells and 83 μg protein = 10^6 cells, for pachytene spermatocytes and spermatids, respectively (from L. J. Romrell's unpublished data).

Figure 3-5. Age Dependent Variation in Synthase Activity in Sertoli Cells, Spermatogenic Cells and Protease Treated Seminiferous Tubules.

Synthase activity was measured under standard conditions in sonicates of Sertoli cells (o), spermatogenic cells filtrate from protease treated seminiferous tubules (Δ), and protease treated seminiferous tubules (•) prepared from rats of different ages.

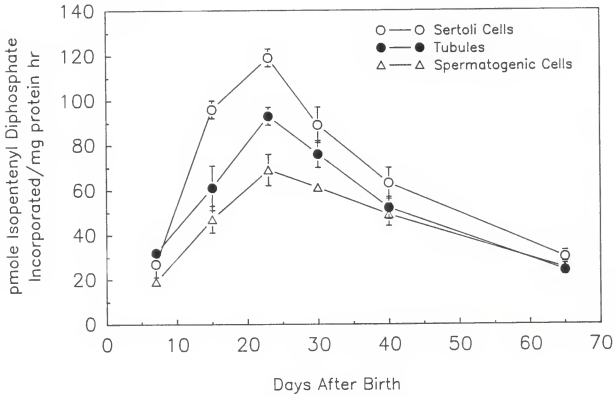

pmole Isopentenyl Diphosphate
Incorporated/mg protein hr
140
120
100
80
60
40
20
0
0
10
20
30
40
50
60
70
Days After Birth
Sertoli Cells
Tubules
Spermatogenic Cells

Table 3-2

Estimated Specific Activities of Dehydro DOL PP Synthase in Pure Spermatogenic Cells[a]

Spermatogenic Cell	% Cell Purity	Specific Activity (pmoles/mg protein) Enriched	"Pure"
Spermatogonia	100	19	19
Pachytene Spermatocytes	80	91	100
Spermatid	85	64	58
Spermatozoa	100	12	12

[a]Specific activities for pachytene spermatocytes and spermatids were estimated by assuming that each of these populations was contaminated by the other cell population. The two simultaneous equations $SA_{Enriched}$ = Fraction Pachytene (SA_{Pach}) + Fraction Spermatid(SA_{Sper}) for the enriched populations of spermatocytes and spermatids were solved to obtain the specific activities of both pure cell populations.

Table 3-3

Estimated Specific Activities of Dehydro DOL PP Synthase in "Pure" Sertoli Cells From Rats of Different Ages[a]

Day of Age	%Purity of Sertoli Cells	Specific Activity (pmoles/mg Protein) Enriched Sertoli Cells	Enriched Spermatogenic Cells	"Pure" Sertoli Cells
7	87	27	19	28
14	88	109	47	118
23	88	119	69	126
30	86	89	61	94
40	85	63	49	66
65	81	30	25	29

[a]Specific activities of "pure" Sertoli cells were estimated by assuming that each of the enriched Sertoli cell populations was contaminated by the same mixture of spermatogenic cells as that isolated from rats of the same age. The following equation $SA_{Enriched\ Sertoli}$ = Fraction Sertoli cell ($SA_{Pure\ Ser.}$) + Fraction Germ cell ($SA_{Enriched\ germ}$) was solved to obtain the specific activity for each "pure" Sertoli cell population.

Discussion

Glycoproteins are undoubtedly critical components in the spermatogenic process. The timing of their elaboration is thought to be critical for spermatogenesis to proceed normally. Histochemical studies in spermatogenic cells show that the Golgi apparatus of spermatocytes and early spermatids are highly active in glycoprotein biosynthesis (Letts et al., 1974b). However, the Golgi apparatus degenerates and is lost from spermatogenic cells as spermatids mature into spermatozoa. At least some of the enzymes necessary for N-glycoprotein biosynthesis are presumably lost as well. Letts et al (1974a) have shown developmental dependent changes in the specific activities of three glycosyl transferases involved in the late reactions of oligosaccharide maturation in glycoprotein biosynthesis. Galactosyl and N-acetylglucosaminyl transferases were found in spermatogonia, whereas the fucosyl transferase was not highly active until the spermatocytes appeared. All three enzymes were low in spermatozoa. A later study (Letts et al., 1978) indicated that spermatocytes and early spermatids were highly active in glycoprotein biosynthesis. Therefore, it was of interest to investigate if other biological machineries necessary for the glycoprotein biosynthesis are changing accordingly in these cell populations.

The timing of the secretion of glycoproteins such as androgen binding protein and plasminogen activator by the Sertoli cell, has been described in several laboratories (Parvinen, 1982). Furthermore, there are now numerous reports which show that the secretion of androgen binding protein may be regulated by the type of spermatogenic cells associated with the Sertoli cell at different times during the spermatogenic cycle (LeMagueresse et al., 1980; Ritzen et al., 1982; Galdier, 1984).

Recent studies indicated that the mammalian testis exhibits unusually high rates of DOL synthesis. This could be related to high rates of glycoprotein biosynthesis, and to temporally regulated synthesis of acrosomal enzymes in late pachytene spermatocytes or early spermatids (James & Kandutsch, 1980c; Wenstrom & Hamilton, 1980; Potter et al., 1981b). Acrosomal enzymes may also represent end products of dolichol-mediated glycosylation in the testis since many of these constituents are glycoproteins (Flechon, 1979; Mukerji & Meizel, 1979). Since DOL P has such a critical role in N-linked glycoprotein biosynthesis, its availability is also a potentially regulatory factor of glycoprotein biosynthesis during spermatogenesis. The results of previous work (Allen & Ward, 1987; Chapter II) showed that the level of DOL P and dehydro DOL PP synthase increased in parallel between day 7 and day 23 of spermatogenesis in the seminiferous tubules of immature rats. Synthase activity then decreased to a level

at day 60 near that seen at day 7. The increase in DOL P levels is consist with the observations of Nyquist and Holt (1986), who showed an increase in DOL concentrations in rat testes during this time period and the work of Potter et al. (1981b), who showed a high rate of DOL biosynthesis from acetate by mouse pachytene spermatocytes. Potter and coworkers also showed that hydroxymethyl glutaryl CoA reductase activity was high in pachytene spermatocytes. Nyquist and Holt (1986) have also reported that DOL concentration was high in Sertoli cells and suggested that DOL may be synthesized in the spermatogenic cell then transported to and accumulated in the Sertoli cell. This conclusion was supported by the observation by James and Kandutsch (1980c) that testes of x-irradiated mice or testes of mutant mice severely deficient in spermatogenic cells (but with apparently normal Sertoli cells) incorporated acetate into DOL at a 20 fold lower rate than normal testes.

However, the results reported here show that both the spermatogenic cells and the Sertoli cells contribute substantially to the dehydro DOL PP synthase activity of rat seminiferous tubules during early stages of testicular development. This is not surprising considering the active glycoprotein biosynthesis occurring in both cell types, the rapid changes occurring in the spermatogenic cell during differentiation and the active role of the Sertoli cell in supporting this development.

Assignment of the relative contribution of the spermatogenic cells and the Sertoli cells to the changing synthase specific activities at different times during early spermatogenesis requires an assessment of the fraction of each of these cells present at different ages and knowledge of the specific activities of the "pure" cell types. An interpretation of these changes is made here on the basis of the reported results (Fig. 3-6).

At 7 days of age the synthase activities of both the Sertoli and spermatogenic cells are low. At this stage of differentiation the Sertoli cells are still dividing and the spermatogenic cells have not yet reached the meiotic phase. It is reasonable that the observed synthase activity from testes at this age was low.

At 15 days of age, the Sertoli cells have stopped dividing (Steinberger and Steinberger, 1971) and at least some of the spermatogenic cells have entered the meiotic phase. Dehydro DOL PP synthase in spermatogenic cells has increased but the cell number is small. Therefore, spermatogenic cells do not contribute in a major way to the total specific activity of the synthase in the whole tubules. In contrast the Sertoli cells are relatively high in both number and in enzyme specific activity. Therefore, the total tubular synthase activity is mainly due to Sertoli cells.

At 23 days of age, the enzyme specific activities of both early pachytene spermatocytes and Sertoli cells have

peaked. The number of pachytene cells relative to other spermatogenic cells is maximum, and there are few if any spermatids present by this time. The number of Sertoli cells has become relatively constant. Therefore, the total activity of synthase is due to the sum of Sertoli cell, pachytene spermatocytes and other spermatocytes preceding the pachytene stage. The specific activity is optimal at this time, because Sertoli cell specific activity is highest at this point and the relative percentage of pachytene spermatocytes (the spermatogenic cell with the highest specific activity) is also highest at this time of development. Actually, it is the only time period in the rat's life span that the relative percentage of pachytene spermatocytes reaches a peak value (Fig. 3-6).

At 30 days of age, the pachytene spermatocytes still have active synthase activity but the relative number of these cells present in the seminiferous tubules is a smaller fraction of the total spermatogenic cell population than at day 23. The Sertoli cell number is constant but its synthase specific activity has decreased by day 30. The contribution of spermatids to the total activity is relatively low at this time, since the number of spermatids is small although increasing rapidly. Our data also indicate that as pachytene spermatocytes differentiate into spermatids, the enzyme specific activity decreases by 30 % (Fig. 3-4). There was, however, no net change in total enzyme activity during the

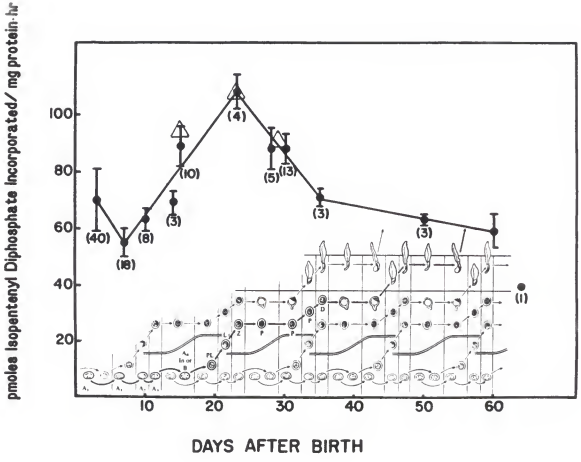
division of pachytene spermatocytes into spermatids (Table 3-1). Most of the enzymatic activity is lost during the differentiation of spermatids to spermatozoa. Therefore, the decrease in tubular synthase activity after 23 days can be accounted for by the decreasing fraction and specific activity of Sertoli cells and the fact that the pachytene spermatocytes, which have the highest spermatogenic cell specific activity, are becoming a smaller fraction of the total spermatogenic cells population and are being replaced by spermatids with lower specific activity.

Kumari and Duraiswami (1986) have estimated the percentages of Sertoli and spermatogenic cell populations in rat seminiferous epithelium at various days during early stages of spermatogenesis. If these percentages are used in conjunction with the enzyme specific activities observed here for the Sertoli and mixed spermatogenic cell population, the predicted enzymatic activities of the whole tubular homogenate can be calculated for animals 15, 23, and 30 days of age (Fig. 3-6). There was a good correlation between the predicted specific activity and the observed activity from this study except for the result on day 15.

In general, the specific activity of dehydro DOL PP synthase in Sertoli cell was always higher than that in spermatogenic cells measured between day 7 and day 65 of age. This suggests that glycoprotein biosynthesis is more active in Sertoli cell than in spermatogenic cells. Kumari and

Figure 3-6. The Relationship between the Dehydro DOL PP Synthase Activity and Spermatogenesis during Testicular Development.

The curve of the enzymatic activity of rat testicular development is from the results in the previous chapter. The scheme is modified from B. P. Setchell., 1982, in Germ Cell and Fertilization, Eds. C. R. Austin and R. V. Short, New York: Cambridge University Press, PP. 63-101. The calculated results are shown as (Δ).


pmoles Isopentenyl Diphosphate Incorporated/ mg protein-hr
100
80
60
40
20
10
20
30
40
50
60
DAYS AFTER BIRTH
(40)
(18)
(8)
(3)
(10)
(4)
(5)
(13)
(3)
(3)
(1)

Duraiswami (1986) have concluded, based on an autoradiographic study, that the protein synthetic potential of Sertoli cells is greater than that of spermatogenic cells at any stage of differentiation. The parallel results seen between that study and results represented here may be highly significant and suggest a key role for dehydro DOL PP synthase in regulating glycoprotein biosynthesis in Sertoli cells during spermatogenesis in rats.

The reason for the rise and fall of the enzyme specific activity in Sertoli cells in the immature testes is not well understood. Enzyme activities and protein secretion in rodent seminiferous tubules have been shown to peak at about day 20 to day 23 in several cases (Parvinen,1982). This has been attributed in some cases to the appearance of pachytene spermatocytes with their constituent enzymes and in other cases to the onset of new protein synthesizing activities in Sertoli cells. In the current study, the results showed that both Sertoli cell and spermatogenic cells are contributing to the increase of the dehydro DOL PP synthase activity. The possibility that the presence of the pachytene spermatocyte may cause an increase in Sertoli cell synthase activity is an interesting conjecture, which has been postulated for other systems (LeMagueresse & Jegou, 1986; Ireland & Welsh, 1987) and requires further study.

CHAPTER IV
CONCLUSIONS AND DIRECTIONS

Dehydro DOL PP synthase, which catalyzes the synthesis of dehydro DOL PP from farnesyl diphosphate and isopentenyl diphosphate could be very important for controlling DOL P level in eukaryotic cells, since this is the only de novo biosynthesis pathway known and serve as a "bridge" connecting the small metabolites, such as acetyl CoA, with the large DOL molecules. Therefore, dehydro DOL PP synthase could be an important cellular regulator of glycoprotein biosynthesis as a consequence of its regulation in the DOL P de novo biosynthesis.

An attempt has been made to further characterize the enzymatic products, namely, dehydro DOL PP and dehydro DOL P. The precursor-product relationship between dehydro DOL PP and dehydro DOL P seems clearly established.

It has been reported that the level of DOL P, a carbohydrate carrier in glycoprotein biosynthesis, is regulated during spermatogenesis (James & Kandutsch, 1980b; Potter et al., 1981b). Temporal expression of seminiferous tubular dehydro DOL PP synthase has been shown to correlate well with the increase in Dol P during early stages of differentiation in prepuberal rats. The results presented in

Chapter II support the hypothesis that increasing DOL synthesis observed during testicular development (Potter et al., 1981b) is due at least in part to an increase in the dehydro DOL PP synthase activity.

The cellular localization of this increased synthase activity was of interest because of the multicellular nature of the testicular tubules. The specific activity of synthase in homogenates of protease treated seminiferous tubules, cell fractions enriched in spermatogenic cells or Sertoli cells peaked in rats aged 23 days, as shown with non-protease treated cells. Homogenates of cell fractions enriched in pachytene spermatocytes, spermatids or Sertoli cells had higher synthase activity than a whole testicular homogenate or a mixture of cells prepared by protease treatment of tubules. Enzymatic activity in pachytene spermatocytes expressed per mg protein, was about 5.3 fold higher than spermatogonia, 1.7 fold higher than in spermatids and about 8.3 fold higher than in spermatozoa. Therefore, the increase of the synthase activity in spermatogenic cell before day 23 can be accounted for by the appearance of the pachytene spermatocytes. Little net increase in enzyme occurred during or after meiotic cell division of pachytene spermatocytes into spermatids. The enzymatic activity decreased remarkably during the differentiation of spermatids into spermatozoa.

It is reported for the first time that Sertoli cells have the potential to synthesize DOL P. The enzymatic

activity in enriched Sertoli cells was 1.5 to 2.3 fold higher than in the enriched spermatogenic cells between day 14 and day 30. The increase in synthase activity in spermatogenic cells and Sertoli cells indicates that both are contributing to changes in the enzymatic activity in seminiferous tubules. In addition, and perhaps more significantly, the work presented here provides evidence that dehydro DOL PP synthase may be important in regulating the availability of Dol P for glycoprotein synthesis during early stages of spermatogenesis in rat.

Future research on the role that dehydro DOL PP synthase plays in spermatogenesis in rat will focus on several fundamental questions. In vitro studies on the incorporation of radiolabeled probe, such as mevalonate, into DOL with enriched Sertoli cells will determine whether synthase activity function in these cells in vivo. Synthase activity would also be measured in co-cultures of Sertoli cells and different spermatogenic cells. This could test the role of cell-cell interaction with respect to the function of DOL P in regulating spermatogenesis. Activators or inhibitors of this enzyme may also be produced as a result of cell-cell interactions. Such questions can now be approached with the techniques and information presented in this dissertation.

APPENDIX A

SUMMARY OF EXPERIMENTAL DATA PRESENTED IN FIGURE 2-13 DEHYDRO DOL PP SYNTHASE ACTIVITY IN SONICATES OF TUBULES FROM RATS OF DIFFERENT AGES.

DATE / AGE	3	7	10	14	15	23	28	30	35	50	60	64
85/1/16				64.5				77.3			51.3	
2/5					82.5			70.7				39.0
4/8				72.9			97.2			66.3		
										62.9		
										59.5		
4/22	85.2											
4/30	50.9	51.2			107.4			106.0			59.9	
5/16	50.4	44.4			90.2			92.7			48.8	
5/24	94.7	71.0			74.9			92.7			74.8	
6/10		34.3							71.0			
		56.3							73.2			
		67.0							63.5			
6/22			58.7				71.7					
			71.5				91.1					
			60.7									
			53.2									
7/3						100.7		73.3				
						119.9		98.1				
						110.0						
						94.1						
$\bar{x}$	70	54	61	69	89	106	87	87	69	63	59	39
S_x	23	14	8	6	14	11	13	14	5	3	12	
N	8	12	8	4	8	8	6	14	6	6	8	2
n	4	6	4	2	4	4	3	7	3	3	4	1
$S_x/\sqrt{n}$	12	6	4	4	7	6	7	5	3	2	6	
Animals	40	18	8	3	10	4	5	13	3	3	10	1

Mean = $\bar{x}$ pmoles/mg protein

Standard Deviation = $S_x/\sqrt{n}$ pmoles/mg protein

Number of Experiments = n

Number of Assays = N

APPENDIX B

TYPICAL NUMBER OF RATS, TOTAL TUBULE WEIGHT, AND NUMBER OF ASSAY USED FOR EACH EXPERIMENT AS A FUNCTION OF RAT AGE

Age (Days)	# of Rats Used	Total Tissue Weight (g)	Assays
3	10	0.09	2
7	4 - 5	0.12	2
15	2	0.20	2 - 3
23	2	0.85	3 or more
30	2	1.20	3 or more
40	2	1.60	3 or more
60	2	3.50	3 or more

APPENDIX C

SUMMARY OF EXPERIMENTAL DATA PRESENTED IN FIGURE 3-5. AGE DEPENDENT VARIATION IN SYNTHASE ACTIVITY IN SERTOLI CELLS, SPERMATOGENIC CELLS AND PROTEASE TREATED SEMINIFEROUS TUBULES. SYNTHASE ACTIVITY IS PRESENTED IN UNITS OF PMOLES ISOPENTENYL DIPHOSPHATE INCORPORATED/MG PROTEIN

Age (Days)	Rats	Assays	Sertoli Synthase Activity	Rats	Assays	Tubules Synthase Activity	Rats	Assays	Spermatogenic Cells Synthetase Activity
7	24	4	27 ± 6	12	4	32 ± 2	24	4	19 ± 2
15	12	4	109 ± 4	12	6	61 ± 14	12	4	47 ± 7
23	7	4	119 ± 4	7	6	93 ± 4	7	4	69 ± 2
30	4	4	89 ± 8	4	6	76 ± 7	4	4	61 ± 2
40	6	4	63 ± 7	6	4	52 ± 6	6	4	49 ± 5
60	4	4	30 ± 3	4	4	24 ± 1	4	4	25 ± 3

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BIOGRAPHICAL SKETCH

Zhong Chen was born in Beijing, China, in 1944. In 1962, Zhong began his premedical education at Peking University. Later Zhong studied medicine at China Medical College (the former Peking Union Medical College founded by the Rockefeller Foundation) and he received the M.D. degree in 1968. After graduation he practiced medicine for several years and specialized in ophthalmology. In 1981, Zhong visited the Royal Hospital (Rigshospitalet) and the University of Copenhagen in Denmark as a visiting scholar in the Departments of Ophthalmology and Virology. During that period of time, Zhong realized that biochemistry and molecular biology are the keys to open the mysterious kingdom of medicine. So he decided to brush up on biochemistry and use it as a tool to explore the unanswered questions in medicine.

After finishing his Ph.D., Zhong will move to Oklahoma City, Oklahoma, and the laboratory of Dr. Jordan J. N. Tang in the Oklahoma Medical Research Foundation to continue his training.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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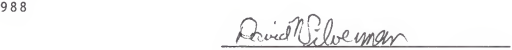
April, 1988



Dean, College of Medicine

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